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THE UNIVERSITY OF ALBERTA

THE SIGNIFICANCE OF DNA BASE RATIOS  
IN BACTERIAL TAXONOMY

by

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A THESIS

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## ABSTRACT

Base ratios of various bacteria were determined. The first group of organisms studied comprised various Gram-negative obligately or facultatively anaerobic 'corroding' bacilli. The base ratios of these organisms fell into three distinct groups; the facultative anaerobes having 56.9%-57.9% GC, two strains of obligate anaerobes having 38%-39% GC, and the remaining strains of obligate anaerobes having 28.0%-29.7% GC. Base ratios of other Gram-negative facultative or obligate anaerobes were also determined and the relationships of these organisms with the corroding bacilli were investigated using a computer analysis of their base ratios and biochemical characteristics. The results of the analysis showed all the facultatively anaerobic corroding bacilli, with base ratios of 56.9%-57.9% GC to be located in one cluster, distinct from the obligately anaerobic strains with base ratios of 28.0%-29.7% which were all located in a second cluster and from strains of Vibrio (Campylobacter) which occurred in a third cluster. The remaining OTU's formed single member clusters, and two obligately anaerobic strains of corroding bacilli which had base ratios of 38%-39% GC appeared to be quite closely related to species of the genus Bacteroides. The desirability of two new genera to accommodate the corroding bacilli with base ratios of 56.9%-57.9% GC and 28.0%-29.7% GC is suggested.

Base ratios of some organisms classified as Micrococcus species which had previously been subjected to computer analysis were determined. Organisms which were classified as species of Micrococcus but were put in clusters with species of Staphylococcus on computer analysis, and had





Staphylococcus-like cytochrome patterns, were found also to have Staphylococcus-like base ratios, with the exception of M. cyaneus CCM 856.

Base ratios were determined for a number of strains of Pasteurella multocida, and for other species of Pasteurella. The values obtained for strains which were serologically distinguishable were too similar to allow the serotypes to be distinguished by their base ratios, and only P. pseudotuberculosis (proposed to belong to the genus Yersinia) had a significantly different base ratio from the other strains studied.





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## LIST OF ABBREVIATIONS

|                |                                        |
|----------------|----------------------------------------|
| A              | - adenine                              |
| C              | - cytosine                             |
| °C             | - degrees Centigrade                   |
| DNA            | - deoxyribonucleic acid                |
| DNase          | - deoxyribonuclease                    |
| EDTA           | - ethylenediamine tetra acetic acid    |
| Fig.           | - figure                               |
| G              | - guanine                              |
| % GC (GC)      | - guanine + cytosine base pairs in DNA |
| <u>M</u>       | - molarity                             |
| ug.            | - micro-gram                           |
| nm.            | - nanometer                            |
| OTU            | - Operational Taxonomic Unit           |
| RNA            | - ribonucleic acid                     |
| RNase          | - ribonuclease                         |
| SSC            | - standard saline citrate              |
| T              | - thymine                              |
| T <sub>m</sub> | - melting temperature                  |
| % (w/v)        | - percent weight by volume             |





## INTRODUCTION



## INTRODUCTION

In biology the arrangement of data is essential for the correlation of accumulated facts about a variety of subjects and organisms; a nomenclature which is precise, unequivocal and clearly defined is equally important for the communication and reference of data. Thus taxonomy, as the science or art of classification and nomenclature as usually applied to living organisms is an essential feature of the scientific method, and as such, should serve the dual purpose of distinguishing between species, and demonstrating the relationships between them.

The principles of taxonomy as laid down by Linnaeus, served as the basis for the classification and nomenclature still used in botany and zoology, and it was inevitable that the first attempts at classification of microorganisms should follow this system. Classification being based on observable characters develops with the techniques for recognition of these characters. Hence an initial classification based on morphology alone was superceded by a system which added biochemical and physiological considerations to those of shape and arrangement of parts. However the value of a taxonomy can only be estimated in relation to the function which it is required to perform, and since the prevalent interest in bacteriology has been in clinical diagnosis, classification of bacteria has developed as a catalogue for identification, rather than a taxonomy based intrinsically on the organisms it seeks to classify. The resultant Linnaean nomenclature has emerged as confused and ambiguous, partly through various authors calling one





organism by different names, and partly through the attribution of the same name to different organisms. The majority of microbial classifications are based on only a few of the characters of the organism they seek to identify, the selection of these characters being influenced by their practicability in routine diagnostic tests rather than by any intrinsic significance. Not only are these characters selected on purely extrinsic grounds, but the sequence in which they are examined may determine which features are subsequently investigated. Lack of standardization of methods employed in diagnostic tests, and a lack of their quantitation, also leads to irregularities in reports of properties of bacteria; a result which is positive in one test or with one reagent concentration may prove negative by another test or with organisms grown under different conditions. Standardization is therefore necessary before any Adansonian analysis of characters can be meaningful.

A classification would analyse all the characteristics of an organism if its object were to reflect natural interrelationships, a principle put forward by Adanson in 1765. Sneath (1957) contended that scientific classification should attempt to measure overall similarity, which would result in a high predictive value for such a taxonomy. The ideal taxonomic system therefore would be the one containing the greatest amount of information. The numerical evaluation of similarities between microorganisms, using as many characters as possible, results in a numerical taxonomy, and logically overall similarity is measured in terms of the number of similar characters possessed by two organisms, all characters being independent and having equal weight. This may give rise to polythetic or monothetic groupings. In polythetic grouping no



one organism necessarily has all the features characteristic of the group, and the group members do not necessarily all share any common property, the degree of relationship being expressed as the taxonomic distance which is a function of the similarity ratio. Analysis at given levels of similarity may result in "clusters" of OTU's (Operational Taxonomic Units). In this cluster analysis, single linkage groups have the disadvantage that inclusion within the cluster requires only that an organism attain the given level of similarity with any one of the pre-existent members of the cluster: the level of similarity between members of the cluster may be very low. Linkage between clusters occurs at that level of similarity at which two organisms in separate clusters at higher levels occur in the same cluster. Thus a multilinkage cluster is one in which every incorporated OTU agrees with every other OTU in the cluster at a level of similarity which is not less than that at which the multilinkage cluster occurs. Monothetic hierarchical groups present a system in which addition of a new member is determined by its possession of a spectrum of properties common to every pre-existent member of the group, and these relationships can be expressed in terms of cumulative difference.

Only with the advent of computer analysis has the evaluation with equally weighted characters become feasible, but the selection of these characters is still limited even if only by ignorance of the metabolic capabilities of the organisms. Also it is difficult to know from biochemical manifestations which characters are unrelated and independent, and to avoid placing undue emphasis on what is intrinsically a single characteristic. For example, an organism lacking the ability to trans-





port  $\beta$ -galactosides across its membrane will be judged to be non-lactose fermenting; however the same enzymic deficiency will result in the organism's being unable to transport galactose, inulin, fructose etc., and if these characters be given equal weighting, undue weight will be given to what is basically a single character. Conversely if these characters are not scored individually, and the organism's inability to ferment a variety of sugars be not due to lack of a common enzyme but to a lack of a number of specific enzymes, significant variations will be ignored.

Specific differences can only be made to demonstrate what is derivative from the "essence" of the species, and cannot be used to demonstrate essence or definition itself. Perhaps the nearest definition with which science at the moment can approach to "essence", lies in the genetic material of organisms, the uniqueness of which may distinguish one species from any other. In zoological classification, one of the primary criteria for judging animals to be related at the species level is their ability (or in the case of geographically isolated populations, their potential ability) to interbreed. This ability is a function of the genetic homology which exists between members of the same species. Since bacterial reproduction is usually asexual, interbreeding cannot be applied as a necessary criterion of genetic similarity, but in certain species, genetic transfer has been observed in the processes of transformation, transduction, and conjugation. In cases of transformation, interstrain crosses have been extended to interspecies, and even to intergeneric levels, because the amount of genome being transferred is very small, and the chances of its being homologous with some portion of the recipient genome are correspondingly increased. In transduction,





interspecies transduction is limited by the host range of the transducing 'phage, and no examples of transduction between different species have been reported (Marmur, Falkow and Mandel, 1962). In conjugation, a part of the donor genome is transferred to the recipient, the size of the exogenote being dependent on the duration of contact between the two bacteria. Interspecific crosses between Escherichia coli and strains of Salmonella and Shigella species result in recombinants being formed, but at a rate which is some thousand times lower than the rate of recombination in crosses between two strains of E. coli. Crosses between Salmonella and Shigella strains produce recombinants at a frequency a thousand times less than that shown by either species with E. coli, and in addition, the recombinants formed between Salmonella and Shigella species are unstable, which indicates that the homology of genome between these two species is less than the homology of either with E. coli. The genotypic level would therefore appear to be the level at which the species can, at the moment, be most fundamentally defined. The individuality of the genome lies in the sequence of the purine and pyrimidine bases, adenine (A), guanine (G), cytosine (C), and thymine (T) in the deoxyribonucleic acid of the 'chromosome'. The sequence of these bases has been shown to constitute the 'genetic code'. Read in groups of three, each triplet of bases forms a specific codon for one amino acid. The code on the DNA, by mediation of m-RNA and t-RNA is transcribed and translated into a colinear sequence of amino acids to form the proteins of the cell as determined by the genome (Yanofsky, 1967). However techniques for determination of base sequences are not yet sufficiently developed for routine purposes, though other properties of DNA can be



used to give an estimate of the proportions of bases present. Although organisms with the same base content are not necessarily related, e.g. Pseudomonas aeruginosa and Mycobacterium phlei both have 66% of their bases as guanine+cytosine (GC), and Clostridium nigrificans and Pasteurella pestis both have 46% GC, organisms with widely different base ratios are unlikely to be related. The most important application therefore of the estimation of base ratios to taxonomy would appear to be in the distinguishing of species which have very similar biochemical reactions, or are biochemically relatively inert, but are of widely different GC content.





## II

### LITERATURE REVIEW



## LITERATURE REVIEW

The possibility of an approach to the taxonomy of microorganisms based on the chemical composition of their genetic material arose from the isolation of DNA from bakers' yeast by Chargaff and Zamenhof in 1948, and the subsequent analysis, identification and quantification of the bases present by Vischer and Chargaff (1948). Evidence was presented (Chargaff, 1949) that DNA from different organisms differed in base composition, when DNA extracted from tubercle bacilli was found to have significantly higher content of guanine and cytosine than that previously extracted from yeast. Other species of bacteria were examined by laborious techniques taking seven days for the extraction of the DNA, and its analysis by chromatography. In all cases DNA from individual strains of one organism demonstrated a constant base composition, regardless of the age of the culture or conditions of growth etc., and this ratio was found to be different from that of other species. Chargaff (1956) described DNA from several species, all of which were of the AT type, having more adenine and thymine than guanine and cytosine. A few GC type organisms were noted by later workers and Chargaff pointed out that the purine and pyrimidine bases were always present in equimolar amounts. The first intermediate values for base ratios were reported by Gandelman et al. (1952) using three strains of Escherichia coli. The GC contents of all three strains were found to be within 1% of each other, demonstrating the specificity of the base ratios within a species. Since then many organisms have been investigated, by several different techniques. The identification of bases by chromatog-



raphy, and the estimation of phosphorus content for their quantification used by Chargaff has been largely superseded by techniques dependent on the physico-chemical properties of the DNA itself. Sueoka (1959) analysed the distribution of DNA in caesium chloride density gradient ultracentrifugation, and the linear relationship between GC content and buoyant density was confirmed by Schildkraut, Marmur and Doty (1962) as

$$\rho = 1.660 + 0.098(\text{GC}).$$

Spectral analysis of DNA has been used by Fredericq, Oth and Fontaine (1961) to determine base ratios, but highly purified DNA is required for this method. One of the most convenient methods for determination of base ratios is that of  $T_m$  analysis first suggested by Marmur and Doty in 1962. However Gasser and Mandel (1968) report considerable discrepancies in values of base content reported for the same organism if determined by different methods, and they say that  $T_m$  analysis may in some cases give GC content up to 5% lower than those reported by other methods of determination.

A further method of determining the relatedness of bacterial species is the estimation of the degree of homology shown by their DNA in annealing experiments. This procedure was first described by McCarthy and Bolton in 1961 and has since been used in DNA-DNA and DNA-RNA hybridization experiments in many species.

The first review on the subject of base ratios was published by Belozersky in 1956, and subsequent reviews have been supplied by Marmur, Falkow and Mandel (1962), Hill (1966) etc.

The grouping of organisms according to their base ratios agrees to a large extent with the classification of bacteria as set out in





Bergey's Manual of Determinative Bacteriology (1957), which is based on considerations of morphological and biochemical similarity. Data are not available for many of the species listed in Bergey, and only the Schizomyces have been studied in any detail with regard to the taxonomic significance of their base ratios.

Of the order Pseudomonadales, suborder Rhodobacterineae, few species have been investigated. In the family Thiorhodaceae, the purple sulphur bacteria, values for the genus Chromatium of 64%-66% GC have been reported (Marmur, Falkow and Mandel, 1962. (79)\*).

Of the family Athiorhodaceae, the non-purple sulphur bacteria, only Rhodospirillum rubrum has been studied and values of 60%-67%GC reported (79).

The family Chlorobacteriaceae has been investigated for base ratio values only in the genus Chlorobium, which is divided by Bergey into two species: Chlorobium limicola which cannot utilize thiosulphate as an oxidizable substrate and Chlorobium thiosulphatophilum, which can. In studies by Mandel, Bergendahl and Pfennig (1965) of 17 members of the genus, three subgroups of organisms were indicated on the basis of their GC content. These groups had 52%, 53%-55%, and 57%GC, which the authors suggest to correspond with three species of the genus. However the ability to utilize thiosulphate is not peculiar to any one of these groups, suggesting that perhaps this criterion is trivial and not one valid for interspecies differentiation.

\*References to review articles will be made by name and number in the first instance, and subsequently by number only, to avoid repetition.



In the second suborder, Pseudomonadineae, family Nitrobacteriaceae, only one species of the genus Nitrosomonas has been studied for base ratios, and this organism was found to have a GC content of c. 55% (79). Only the genus Thiobacillus of the family Thiobacteriaceae has received any attention in respect of base ratios. Two species, Thiobacillus thioparus, and Thiobacillus novellus, distinguished by Bergey in that the former is strictly anaerobic, and the latter only facultatively so, were shown (Belozersky, 1960 (4);(79)), to have widely different base ratios of 69% GC, and 58% GC respectively.

In the family Pseudomonadaceae, the genus Pseudomonas has been widely studied, and most of the organisms included in the genus by Bergey have been shown to have base ratios of 61%-68% GC. An analysis of the morphological and biochemical characteristics of the group by Stanier, Palleroni, and Doudoroff (1966), was augmented by a determination of the base ratios of those strains studied, by Mandel (1966). The groups studied showed the fluorescent strains to fall into three homogeneous biotypes with a GC content of 62.7% corresponding to Pseudomonas aeruginosa, 59%-63% GC corresponding to Pseudomonas fluorescens, and 61% GC being found in Pseudomonas putida. The acidovorans group showed two biotypes among 26 strains; one having 66.8% GC and the other having 61.8% GC. Of the seven strains of the alcaligenes group which were studied, six strains of Pseudomonas pseudoalcaligenes had 62.8% GC while Pseudomonas alcaligenes had 55.3% GC. 12 strains of Pseudomonas multivorans had 67.6% GC, and 17 strains of Pseudomonas stutzeri showed two biotypes which had 62.1% GC (proposed as a new species, Pseudomonas stanieri), and 65% GC. Pseudomonas maltophila which was examined by





Stanier in 23 strains, was shown to have a GC content of 66.9% in the four strains examined by Mandel. Strains of Pseudomonas mallei and Pseudomonas pseudomallei had base ratios ranging from 69.0%-69.5% GC. Other workers have also found consistency to within 4% GC in species of this genus, however certain species show base ratios lower than those of the rest of the genus. Pseudomonas fragi had a GC content of 58% (Hill, 1966 (58)), and three species, Pseudomonas atlanticum, Pseudomonas denitrificans and Pseudomonas marginalis had between 55%-58% GC. An organism classified as Flavobacterium piscicida by Bergey, and as Pseudomonas piscicida by Bein had a GC content of only 44%, which resembled reported base ratios for neither genus. Adansonian analysis of 150 characters of strains of this species showed an 80% similarity between members of the species and a 67% similarity between these strains and some few other members of the genus Pseudomonas, including Ps. atlanticum, Ps. marginalis etc. Pseudomonas cruciviae also had a very low GC content of 40%, in comparison with the rest of the genus. The second genus of the family, the genus Xanthomonas contains species whose base ratios range from 66%-68% GC, and Adansonian analysis of the characteristics of the genus also reflects its homogeneity (DeLey, 1963).

The third genus, Acetobacter has been studied in great detail by DeLey (1963,1964), who showed a continual gradation of base ratios among species and strains of the Acetobacter aceti biotype, from 55.5%-65% GC. This gradation of GC content was found to also correspond to a decrease in the enzymic equipment of the strains. The same author reported the similarity of the A. aceti biotype to the Glucono-



bacter strains showing GC contents for these organisms of 60.3%-63.4% (DeLey, 1964) which range is within that of the A. aceti biotype.

Therefore the author stresses the close relationship of the two biotypes and suggests that both biotypes contain strains without species differentiation.

The genus Aeromonas contains organisms which show base ratios of 56.5%-62.5% GC, with the exception of Aeromonas shigelloides which has a base content of 51% GC. Sebald and Véron (1963) suggest the elimination of this organism from the genus, and since for various reasons it cannot be included in the genera Vibrio or Pseudomonas, nor in any of the genera of the family Enterobacteriaceae, they suggest a new genus Fergusonia be created to include one species, Fergusonia shigelloides.

No data are available on the next seven genera of the family, but the twelfth genus, Halobacterium, has been studied by Joshi, Guild and Handler (1963). These workers reported the presence of two species of DNA in Halobacterium salinarium, and in Halobacterium cutirubrum. In a caesium chloride density gradient, DNA from one band had a GC content of 64%-68%, while that in the other band had 55%-58% GC, in both species of Halobacterium. Other species and strains studied showed base ratios which fell into two groups; one with GC contents of 64%-68%, and the other with a GC content of 55%-58%. However since no further reports of two species of DNA in one organism have been published, the possibility of an initially mixed culture cannot be ruled out.

Family V, the Caulobacteriaceae comprises the stalked bacteria. The genus Caulobacter was analysed on the basis of 78 biochemical tests by Poindexter (1964), since its position in Bergey has varied from an





order, to a suborder, to a genus. The base ratios of the species within the genus vary from 62%-67% GC, with the exception of Caulobacter leidyi which has a base ratio of 55% GC. Since this organism is also rejected on the basis of the similarity matrix, a genus Asticcaulis was proposed to accommodate it. The other genera of this family, and the family Siderocapsaceae have not been subjected to base ratio analysis. The seventh family of the order Pseudomonadales, is the family Spirillaceae. The genus Vibrio, members of which may be confused during routine biochemical investigations with the genera Aeromonas, Arthrobacter, etc., was the subject of a study by Sebald and Véron (1963). The investigation of the GC content of these organisms helps to elucidate their taxonomy. These workers suggested that only aerobic, or facultatively anaerobic organisms be included in the genus Vibrio. These species are characterized by a GC content of c.47%, e.g. Vibrio comma, Vibrio metchnikovi, Vibrio costiculus etc. One strain of 'Vibrio' examined had a base ratio of 38% GC and was identified as Moraxella lwoffii. The genus Campylobacter was proposed to include the anaerobic, non-fermentative species of Vibrio, e.g. Vibrio foetus, Vibrio bubulus etc. which are characterized by a GC content of 29%-34%. A base ratio of 64% was obtained with species of Comamonas, a genus proposed by Davis and Park (1962) to replace the genus Lophomonas, and accommodate Vibrio Alcaligenes (Lehmann and Neumann, 1931), and differentiates these organisms from the genus Vibrio. Further evidence substantiating the validity of these genera was provided by Basden (1968). Véron and Sebald (1964) placed one strain of an organism classified as Pseudomonas ichtyodermis in the genus Vibrio since it had a base ratio of 44.9% GC.





The second genus in Bergey's classification of this family is the genus Desulphovibrio which was studied by Saunders, Campbell and Postgate (1964), in a report on sulphate-reducing bacteria. The organisms in this group were found to fall into four subgroups. Of sporulating sulphate-reducing bacteria, two groups appeared; Desulphovibrio orientis (Clostridium orientis) which has 42% GC, and Clostridium nigrificans which has 45% GC. Of non-sporulating bacteria which reduce sulphate, Desulphovibrio desulphuricans showed three distinct groups among the 30 strains studied; 11 strains had 60%-62% GC, 13 strains had 54%-56% GC, and six strains had 46%-47% GC. These groupings were not detectable in the classification of Bergey. The genus Methanobacterium represented by Methanobacterium soehngenii and Methanobacterium omelianski are of uncertain status in Bergey; M. soehngenii is placed in the genus Methanobacterium, while M. omelianski, a spore-forming anaerobe, is tentatively placed after the genus Clostridium, although it is different from the organism designated Clostridium omelianski. However Tonomura, Malkin and Rabinowitz (1965), found a base ratio for M. omelianski of 44.2% GC, which is higher than any reported for species of the genus Clostridium. Genus VII in the family Spirillaceae is the genus Spirillum, which contains organisms classified mainly on their morphology, and which showed widely varying base ratios- from 28%-61.5% GC. The only organisms so far reported to show any similarity in base content are Spirillum itersonii, and Spirillum serpens (in certain strains), which have base ratios of 54%-57% GC.

The other genera in this family have not yet been studied with regard to their base ratios; neither have the orders Chlamydobacteriales and



## Hyphomicrobiales.

The fourth order of Bergey is the order Eubacteriales, of which the first family is the Azotobacteriales. The genus Azotobacter shows consistency of base ratios in various strains of three species, values of 55%-57.5% GC being reported (4, 79).

DeLey and Rassel (1965) studied the base ratios of the genus Rhizobium in the family Rhizobiaceae. 20 peritrichously flagellated strains had GC contents between 59%-63%, and 15 strains with sub-polar flagella had GC contents between 61%-65.5%.

Members of the genus Agrobacterium were studied in great detail by DeLey (1964, 1966) and typically show GC contents of 59%-62%. In 30 strains of Agrobacterium radiobacter, Agrobacterium rhizogenes, and Agrobacterium tumefaciens, values of 59%-60% GC were found. A u.v. induced mutant of A. tumefaciens was reported to have a GC content increased by 6% to 65.5% GC. Further studies by DeLey (1966) recommend recognition of three species in the genus Agrobacterium, A. radiobacter, A. rhizogenes, Agrobacterium pseudotsugae. All strains thus far studied of these species have GC contents between 59.5%-62.8%. It was also proposed to include the strain Agrobacterium rubi with A. radiobacter var. tumefaciens, and to remove the species Agrobacterium stellatum, and Agrobacterium gypsophilae from the genus. The members of the genus Agrobacterium were held to be genetically distinct from the members of the genus Chromobacter by Heberlein, DeLey and Tijtgate (1967), who did homology experiments with members of the Rhizobiaceae. They found that Rhizobium leguminosarum and A. radiobacter var. tumefaciens would hybridize very well together, as would A. rhizogenes and





Rhizobium meliloti, while experiments with strains of the genus Chromobacter with members of these genera showed insignificant levels of hybridization. However DeLey (1964) found members of the genus Chromobacter to be very closely related on the basis of Adansonian analysis and GC content. Four strains of Chromobacter lividum had 65.6%-66% GC, and Chromobacter violaceum had 66%-67% GC. Pale blue and unpigmented strains of this latter organism had the same base ratio as the dark blue wild types, indicating only a very small change in the nucleotide sequence of the wild type being required to produce the unpigmented mutants. Such mutants would probably not be recognised as members of the species on the basis of routine tests, but would be placed in the genera Pseudomonas, Achromobacter etc., unless their base ratio was estimated.

The family Achromobacteriaceae comprises five genera, for three of which base ratios have been evaluated. The genus Alcaligenes has been tested in only two instances, and widely different values of 55% and 67%-70% GC found by different authors (Colwell, and Mandel, 1964 (28)). The genus Achromobacter has been studied in only one species, Achromobacter fischeri, which was found to have 40%-42% GC (79). The genus Flavobacterium was analysed by Colwell and Mandel (1964), and species of high coefficient of similarity on Adansonian analysis were found to have very similar base ratios. The genus as a whole appeared to be heterogeneous; seven species had 64%-70% GC (28, 79), and four had 35.5%-40.5% GC (DeLey and van Muylen, 1963), and Flavobacterium aquatile had a GC content of 32.5%, together with a low coefficient of similarity on Adansonian analysis.



The family Enterobacteriaceae has been the subject of much detailed investigation, and its biochemical and immunological properties are well established. The family is divided by Bergey into six tribes, but the basis for these divisions is disputed on the grounds of overall similarity as demonstrated by Kreig and Lockhart (1966), and Brenner et al. (1969). The former workers used a computer analysis of 105 features of twelve genera of the family, and four species of Aeromonas. The strains were sorted into phenetic groups on the basis of the highest link criterion. The genera Enterobacter, Escherichia, Klebsiella, Citrobacter, Arizona, Shigella and Salmonella formed a single cluster with little or no evidence of subdivision into tribes or genera, and these organisms were no more closely related to one another than to the group as a whole. However Brenner et al. could demonstrate no significant hybridization amongst these organisms, except between E. coli and Shigella flexneri, Salmonella typhi and Salmonella typhimurium, and Proteus mirabilis and Proteus vulgaris, all of which pairs showed greater than 80% interspecific DNA annealing.

However the base ratios of the Enterobacteriaceae reflect the homogeneity of the family. The tribe Escherichieae as defined by Bergey, contains the genera Escherichia, Aerobacter, Klebsiella and Paracolobactrum, and Marmur and Doty (1962) have reported base ratios for this group. Many strains of E. coli, Escherichia aurescens, Escherichia intermedium, and Escherichia (Citrobacter) freundii have all been found to have base ratios of 50%-52% GC. Species of the genus Aerobacter were slightly more variable in their base ratios; strains of Aerobacter aerogenes showed values of 54%-57% GC and other species having lower values of





50%-54% GC. Species of the genus Klebsiella so far examined have shown values of 50%-52% GC, with the exception of Klebsiella pneumoniae which has 55%-56% GC (79).

The genus Paracolobactrum comprises organisms, which except for a delayed fermentation of lactose, are very similar to E. coli and A. aerogenes etc. in their biochemical reactions. The strains so far examined have base ratios very similar to the strains which they resemble biochemically, e.g. Paracolobactrum aerogenoides has 54%-55% GC. Gause (1964) described mutants of Paracolobactrum species induced with u.v., which have a GC content up to 18% higher than that of the parent organism.

The second tribe in the family Enterobacteriaceae is the tribe Erwinieae, containing the single genus Erwinia, in which Bergey recognises 15 species. All species subjected to investigation of their base ratios have shown 50%-54% GC, which information was used diagnostically by Fedorova (1964) to confirm that a pathogen of water melons was Erwinia carotovora, having a base ratio of 54% GC.

The tribe Serratia contains five species three of which have been studied with regard to their base ratios. These all have 53%-59% GC, and Serratia marcescens, the most widely studied species has been reported as having a GC content anywhere within this range by various authors (28).

Species of the tribe Proteeae all have GC contents of 37%-42% (Schildkraut, Marmur and Doty, 1961 (98); Marmur and Doty, 1962 (78)), values considerably lower than those of other Enterobacteriaceae, with the exception of Proteus morgani which has 51%-53% GC, a ratio very different from other Proteus species, but very similar to most other members





of the Enterobacteriaceae. Members of the tribe Proteeae are nonlactose fermenting as are Tribe V, Salmonelleae, and Tribe VI Shigelleae. In this criterion they differ from the Escherichiaea etc., but the base ratios of the Salmonelleae and Shigelleae are 50%-55% GC, and the serological distinctions among the species are not reflected in their base ratios. In the case of the Enterobacteriaceae then, it would seem that biochemical and serological techniques are able to serve more efficiently than do base ratios in distinguishing between species, and strains.

The fifth family of the Eubacteriales is the family Brucellaceae, the genera of which, unlike the Enterobacteriaceae show little intergeneric similarity in base ratios. Genus I is the genus Pasteurella, in which four major biotypes have been recognised: Pasteurella septica (multocida), Pasteurella pestis, Pasteurella pseudotuberculosis and Pasteurella tularensis, together with a number of much less clearly defined species. It has been suggested (Meyer, 1958), that only the P. septica biotype should remain in the genus Pasteurella. This species is the causative agent of haemorrhagic septicaemia in a variety of animals, and was for many years classified by reference to its host, e.g. Pasteurella bollingeri (bovisepiticum), Pasteurella avicida (avisepiticum) etc. All these strains are closely related immunologically, and their inclusion in one species is fully justified. 60 strains of P. septica were subjected to numerical analysis by Talbot and Sneath (1960), and their close relationship confirmed. Their relationship to other members of the genus was also indicated, and showed P. septica to be closely related to Pasteurella pneumotropica, Pasteurella pfaffii and Pasteurella novicida, but only distantly related to P.



pestis and to Pasteurella pseudotuberculosis which were themselves closely related. The bovine strains and avian strains of P. septica were found (78) to be c. 37% GC, although higher values have since been reported. Pasteurella haemolytica appears to be closely related to P. septica, although it does not cross-agglutinate with it. However Mraz (1969) has suggested a close relationship between P. haemolytica and Actinobacillus ligniersii, and subsequently he has proposed a new species Actinobacillus haemolyticus to accommodate both species. The base ratios of both species are c. 40%-42% GC. Nucleic acid homologies between the A and T types of P. haemolytica have been investigated by Biberstein and Francis (1968), who found good DNA-RNA hybridization between strains of type A, but except for unilateral hybridization with T-m-RNA, and A-DNA, he found no hybridization between strains of type T, or with P. multocida. Urease positive variants of P. haemolytica were found by Henrikson and Jyssum (1960) and designated P. haemolytica var. ureae, although Jones (1962) considers these organisms sufficiently different from P. haemolytica, and from P. septica to warrant a new species called Pasteurella ureae.

P. pneumotropica has been found by Brennan, Fritz and Flynn (1969) to have a GC content of 42%. The nomenclature of the rest of the genus is very equivocal, and the inclusion therein of P. pestis and P. pseudotuberculosis biotypes, and of the P. tularensis biotype is very doubtful. Belozerski' (1955) reports base ratios for strains of P. pestis of 46%-47% GC, and hybridization experiments using five strains of Pasteurella were carried out by Ritter and Gerloff (1966). These confirmed the existence of distinct subgroups within the genus, and showed





reciprocal hybridization between P. novicida and P. tularensis, while only a slight relationship could be demonstrated between the DNA of these species and that of P. pestis, P. pseudotuberculosis and an organism since designated P. ureae. However the DNA of P. pestis was bound to almost the same extent by its homologous DNA and that of P. pseudotuberculosis, while DNA from P. ureae was significantly bound only by DNA from P. pestis. Philip and Owen (1961) recommend the inclusion of P. tularensis and P. novicida in the genus Francisella, and as early as 1946 van Loghem proposed the genus Yersinia to accommodate P. pestis, and P. pseudotuberculosis. An organism designated Pasteurella X by Knapp and Thal (1968) is mentioned by Mollaret and Lucas (1965) as Yersinia enterocolitica. In the genus Brucella, Brucella abortus has been shown to have 55%-56% GC (78), and Hoyer and McCullough (1968 a,b) found GC contents of 56%-58% in strains of B. abortus, Brucella melitensis, Brucella neotomae and Brucella suis. DNA from all these species was equally effective in competitive homology experiments, while Brucella ovis, which also has a base ratio of c. 58% GC did not bind to the other Brucella DNA. Hoyer and McCullough also found the DNA of Bordetella bronchiseptica to have 66% GC, and Baillie (1969) found the base ratio of Bordetella pertussis to be 64.8% GC.

Marmur and Doty (1962) found values of 39%-40% GC for strains of Haemophilus aegypti, Haemophilus suis, and Haemophilus influenzae, and Rogul (1965) found 42% GC in the DNA of Haemophilus gallinarum.

In the family Bacteroidaceae, genus Bacteroides, only Bacteroides (Ristella) insolitus and Ristella (Bacteroides) clostridiformis have been studied for base ratios. The former was found to have c. 40.5%-43% GC



(4), and the latter to have 31% GC.

In the genus Fusobacterium, Fusobacterium fusiformis has been found to have 31.5% GC, and Fusobacterium polymorphum, to have 60% GC (4). Species of the genus Sphaerophorus were studied by Dowell (1964), who found values of c. 38% for several species.

The family Micrococcaceae has been the subject of much computer analysis and Adansonian analysis (Hubalek, 1969), and of many studies on base ratios (Hill, 1959; Silvestri and Hill, 1965; Auletta and Kennedy, 1966 (1); Rosypal, Rosypalová and Hořejš, 1966 (95); Boháček, Kocur and Martinec, 1967 (8)). The work of all these groups reveals the existence of two distinct biotypic groups in the family corresponding to the genera Staphylococcus and Micrococcus. The quite considerable variation and range in the base ratios reported in the literature for species of Staphylococcus may be due to the variety of techniques used in their estimation (Garritty, Detrick and Kennedy, 1969).

Within the genus Staphylococcus, Hill found natural groups on computer analysis, around Staphylococcus aureus, and Staphylococcus saprophiticus, however Staphylococcus lactis and Staphylococcus afermentans showed no such natural clusters, individual strains of these species differing as much from each other as they did from the other species. The group called Staphylococcus roseus was found to differ considerably on Adansonian analysis from other members of the Staphylococci, and was considered to be better named Micrococcus roseus. The determination of the base ratios of these organisms by Silvestri and Hill revealed the existence of two widely different subgroups corresponding with those groups indicated by Adansonian analysis. The majority of





strains classified as Staphylococcus had base ratios of 30.8%-36.5% GC, and the majority of Micrococcus species had base ratios of 69%-75% GC, together with Staphylococcus strains which also showed aberrant patterns in computer analysis, S. afermentans for example. The other genera of the family indicated in Bergey, the genera Gaffkya and Sarcina have been found to contain organisms whose base ratios are encompassed by one of the two previously mentioned groups. Auletta and Kennedy (1966) found values for two different strains of Gaffkya tetragena of 36% and 64% GC, and proposed their reclassification as Staphylococcus and Micrococcus species respectively. Values for the GC content of Sarcina species vary considerably, and one strain of Sarcina (Sporosarcina) ureae having an intermediate value of 43% has been suggested to be removed from either genus. The majority of Sarcina strains however show Micrococcus-like base ratios of c. 72% GC.

Seven species of Gram-negative cocci of the family Neisseriaceae were investigated by Catlin and Cunningham (1961). They found base ratios of 47%-51% GC for six species of the genus Neisseria, and values of 40%-42% GC for a seventh species, Neisseria catarrhalis. They also found that while the six species with similar base ratios gave inter- and intraspecific transformation, N. catarrhalis gave intraspecific transformation only. Similar experiments by Kingsbury (1967) demonstrated homology between 11 strains of Neisseria by hybridization on millipore filters. This demonstrated three groups in the genus; the first contained Neisseria meningitidis and Neisseria gonorrhoeae; the second included Neisseria perflava, Neisseria subflava, Neisseria sicca, Neisseria flavescens, and Neisseria flava; and the third contained N.





catarrhalis and Neisseria caviae. Hybridization experiments by Kingsbury and Weiss (1968) showed that species of Chlamydia exhibited as much homology with species of Neisseria (except N. catarrhalis) as they did with other species of Chlamydia. The other Gram-negative cocci, the genus Veillonella have so far been shown to have base ratios of c. 36% GC.

Family X in Bergey's Manual is the family Lactobacillaceae, which is divided into two tribes. The tribe Streptococcaceae is classified largely on the basis of biochemical tests characteristic of serologically related species. Lancefield groups A,C,F, and G, and species from the viridans and lactic groups have GC contents of 38%-41%, while the base ratios of the enterococcus group, Lancefield group D are somewhat lower, 33.5%-37% GC. Homology experiments by Weissman et al. (1966) showed the genetic relatedness among Lancefield groups and serotypes. Organisms in different Lancefield groups were shown to be related but could be differentiated from each other, and serotypes within Group A could be distinguished. Streptococcus faecalis was found to be related only distantly to Lancefield groups A and H, while Diplococcus pneumoniae appeared more closely related to these groups. One value is reported for the base ratio of a species of the Pediococcus genus, i.e. 44.5% GC. In the genus Leuconostoc, values for two species were found to be 39%-42% GC (58).

In the tribe Lactobacilleae, genus Lactobacillus, base ratios have been used by Gasser and Sebald (1966) to show two major groups within the genus. The species Lactobacillus helveticus, Lactobacillus jugurti, Lactobacillus salivarius and Lactobacillus acidophilus form an homogen-



ous heterofermentative group with 33%-38% GC.

The GC values of the other species in the genus with the exception of Lactobacillus bifidus, show a range from 41%-52.5% GC, and are homofermentative (genus Thermobacterium), strictly heterofermentative (genus Beta-bacterium), or facultatively heterofermentative (genus Streptobacterium). Species having closely similar base ratios such as L. helveticus and L. jugurti, Lactobacillus bulgaricus and Lactobacillus lactis, L. salivarius, and L. acidophilus, etc. are suggested as suitable for the regrouping of two species into one. L. bifidus was suggested by Sebald, Gasser and Véron (1965) as a new genus Bifidobacterium, since the strains examined formed an homogeneous group with base ratios of 57.2%-64.2% GC and which were anaerobic, milk-coagulating Gram-positive rods. These workers also studied four strains of Catenabacterium which showed two distinct groupings; two strains had base ratios of 45% GC, and the other two gave values of 30% GC, a heterogeneity which was also apparent from their biochemical reactions. The strains of Ramibacterium ramosum thus far examined (4) have had GC contents of c. 30%.

In the eleventh family in the order, Propionibacteriaceae, the first genus Propionibacterium appears to be an homogeneous group with base ratios of 66.5%-70% GC (Sebald, Gasser and Werner, 1964). Base ratios of the other genera in the family have not yet been determined.

The family Corynebacteriaceae was studied by Bouisset, Breuillard and Michel (1963), who found some relation between base ratio and enzymic activity in 60 strains of various species examined for biochemical reactions and base ratios. On this basis they defined five groups within the genus. Group I was characterized by 58%-59% GC, and contained organisms which were non-glucolytic (Corynebacterium pseudodiphtheriae,





Corynebacterium parvum, Corynebacterium equi.): Group II comprised species with base ratios of 54.5%-57% GC, which produced acid from glucose, laevulose, galactose and saccharose (Corynebacterium xerosis, Corynebacterium mitiscommune, Corynebacterium humiferum, etc.): Group III contained organisms which produced acid from the above mentioned sugars, and also from arabinose, and had GC contents of 53%-54%, (Corynebacterium diphtheroides, Corynebacterium michiganensis, Corynebacterium fasciens, etc.): Group IV, organisms in which were characterized by a GC content of 51.5%-52.5%, fermented glucose, laevulose, galactose, saccharose and lactose, (Corynebacterium hoagi, Corynebacterium avidum, Corynebacterium pseudotuberculum, etc.): Group V (Corynebacterium acnes, Corynebacterium pyogenes) showed the same fermentation pattern as Group III but had base ratios of only 48% GC.

In the genus Listeria, only one strain has been investigated - Listeria monocytogenes was found to have 38% GC (58). Several strains of Cellulomonas were found by Jones and Bradley (1964) to have 75% GC. Strains of Arthrobacter globiniformis have been found to have 60%-64% GC (DeLey and Schell, 1963).

The family Bacillaceae is divided by Bergey into only two genera, the genus Bacillus containing the aerobic sporeforming rods, and the genus Clostridium containing the anaerobic sporeforming rods. The single genus Bacillus has been suggested on the basis of biochemical reactions to be comprised of several genera. This is confirmed by analysis of base ratios, which demonstrate at least two natural groupings. Bacillus alvei, Bacillus brevis, Bacillus circulans, some strains of Bacillus cereus, Bacillus megatherium, etc. have 33%-37% GC. Other species,



Bacillus thuringensis, Bacillus laterosporus, Bacillus pumillus and other strains of B. subtilis have 40%-46% GC (McDonald et al., 1963). Again Gause (1965) claimed to be able to increase the base ratio of B. subtilis strains from 39% to 62% GC by u.v. irradiation. Weed (1963) made similar claims with regard to a copper induced mutant of the same species. Members of the genus Clostridium have so far been found to have base ratios from 29%-39% GC (Tonomura, Malin and Rabinowitz, 1965), and these ratios show no correlation with the biochemical criteria on which Bergey relates members of the genus.

The fifth order in Bergey is the order Actinomycetales, the members of which show generally higher base ratios than are found in the majority of bacteria.

Family I is the family Mycobacteriaceae, the base ratios of the members of which were studied by Wayne and Gross (1968), who found them to be bimodally distributed in a way which was not related to the rate of growth of the organisms, a feature by which they are sometimes distinguished. The values for GC content so far established are between 66.5%-73% GC. The family Actinomycetaceae has been studied in the species Nocardia by Jones and Bradley (1964), and values of 64%-72% GC were obtained. The genus Actinomyces studied only in the species Actinomyces propionici, which is not listed in Bergey was found to have a base ratio of 66.5% GC (98). For the family Streptomyces, Frontali, Hill and Silvestri (1965) found base ratios of 67%-74% GC, and while differences between the maximum %GC in the genus is sufficient to distinguish the strains, between these strains there is a continuous progression which makes it impossible to recognise taxonomic divisions on this basis alone. Only one species



of the genus Micromonospora (Micromonospora coerulea) has been examined for base ratio, and this was found to have 72% GC (4).

The next three orders in Bergey have not been investigated for base ratios, and in Order IX, Spirochaetales, two genera from the second family Treponemataceae only have been examined. The genus Treponema, species Treponema pallidum has a base ratio of 34%-37% GC (79). In the genus Leptospira two species have been shown to have values of 34%-42% GC (79). Haapala et al. (1969) showed four distinct genetic groups in this genus; pathogenic organisms having base ratios of 36% and 39% GC, and two groups of the Biflexa biotype, which showed two distinct patterns of hybridization.

Order X Mycoplasmatales, in which there is a single genus, Mycoplasma, has been studied for base ratios in six species, and values of 23%-46% GC have been reported, and include the lowest GC contents yet found among microorganisms. Reich et al. (1966) used homology techniques to investigate the relationships between human strains of Mycoplasma, using DNA-RNA hybrids. The results indicated that serologically distinct species can also be recognised by homology experiments. However with the same techniques applied to Mycoplasma strains from tissue cultures, their DNA was indistinguishable by homology techniques, although it could be differentiated serologically.





METHODOLOGY



## METHODOLOGY

### I Principle of Melting Temperatures of DNA

#### 1) Determination of base ratios.

Several techniques are available for the estimation of base ratios in microorganisms, one of the most convenient of which is the determination of the melting temperature, or  $T_m$  of DNA extracted from the organism. The  $T_m$  is a function of the chemical composition of the DNA, since the two different base pairs, adenine and thymine, guanine and cytosine contribute differently to the stability of the helix of DNA. The interaction in the AT base pair involves only two hydrogen bonds, while that in the GC base pair, involves three hydrogen bonds. Other dissimilarities are also apparent in the properties of the base pairs. Changes in the physico-chemical properties of DNA which occur when it is heated, are due to the transition over a relatively narrow range of temperature, of the molecular form from a double-stranded helix to single-stranded coils. The absorbance of DNA at 260 nm., which represents c. 60% of the sum of the absorbances of its nucleotides, is increased on heating by approx. 40%, due to the disruption of its structure. Since the two species of base pairs contribute unequally to the stability of the molecule, the  $T_m$  depends on the mean composition of the molecule, and organisms having a high content of G+C will require more thermal energy to effect the transition from the low energy double-helix to the higher energy single-stranded coils, since the total number of bonds which must be broken to cleave the molecule is greater





than in an organism having predominantly A+T base pairs. Thus  $T_m$  corresponds to a 50% hyperchromicity when bonded base pairs are broken. Marmur and Doty (1962) derived an equation demonstrating a linear relationship between  $T_m$  and GC content in 0.2 M  $\text{Na}^+$ .

$$T_m = 69.3 + 0.41 (\text{GC})$$

## 2) The effect of ionic concentration on $T_m$ .

Schildkraut and Lifson (1965) investigated the effect of ionic strength on the melting temperature of DNA. The electrostatic free energy of the molecule is related to the electrostatic repulsion between the negatively charged phosphate groups on the exterior of the double helix. DNA shows typical polyelectrolyte behaviour, and the area of influence of the macromolecule increases as the salt concentration surrounding it decreases. Since electrostatic repulsion is calculated as a function of salt concentration and distance between charges, as the counter-ion content decreases, the charges on the phosphate groups are less neutralized and yield more repulsing energy. Therefore less external energy is required to disrupt the low energy double-helical form, and cause its transition to the high energy single-stranded coil form. Thus  $T_m$  decreases with the decrease in counter-ion concentration and does not depend on any specific counter-ion. Lowering of the salt concentration below a certain point can of itself cause separation of the strands of the molecule, and the overall molecular configuration appears to be a function of the ionic strength of the environment (Ross and Scruggs, 1968). Schildkraut and Lifson related  $T_m$  and sodium ion concentration in the equation



$$T_m = 16.6 \log M + 102$$

and this equation combined with that of Marmur and Doty serves to relate the three variables in the form

$$T_m = 16.6 \log M + 0.41 (\theta \text{ GC}) + 84.5.$$

The value of this principle in the evaluation of base ratios of organisms with a very high ratio of G+C, was recognised by Owen, Hill and Lapage (1969).

The practical difficulties involved in estimation of melting points which occur at nearly 100°C are considerable, and the use of a lower sodium ion concentration to lower the  $T_m$  has obvious advantages. However these workers found that plots of  $\theta \text{ GC}$  against  $T_m$  resulted in a linear relationship for each of the sodium ion concentration used, these plots were not parallel, but convergent. They therefore derived an equation to relate the three variables, and also to account for the observed convergence.

$$\theta \text{ GC} = [\tan (70.077 + 3.32 M_g)] (T_m - 175.95) + 260.34$$

where  $\theta \text{ GC}$  is the % guanine + cytosine/total bases:  $T_m$  = temperature (°C) corresponding to 50% of the hyperchromicity;  $M_g = \log M (\text{Na}^+)$  of the buffer used.

The same workers demonstrated the applicability of this relationship to a variety of different buffers, and organisms.

## II Computer-aided Analysis of Biochemical Data

Because of the uncertain taxonomic status of some of the organisms being investigated, their relationships to other organisms morphologically



and biochemically similar were studied and analysed with regard to their relative proximity. Assuming that OTU's (Operational Taxonomic Units) have an individuality which can be divided into different aspects, and that each OTU to be studied can be so divided, giving a one-to-one correspondence between these selected parts or attributes for any pair of OTU's, and that the attributes chosen are a representative sample of all such attributes, then these attributes may be considered valid for the classification of the OTU's. Several assumptions must then be made about the relative information content of each attribute. Sneath's interpretation (1962) of an ideal classification was based on equal weighting of all characters and is only applicable if all characters are equally dependent or are independent. If some attributes are included which are 'more important' than others (a value judgement justifiable only in relation to the function of the particular classification), or contain more information about the OTU's e.g. a test which determines several equally reliable alternatives gives more information than a test which allows only negative-positive interpretation, weighting of these characters in proportion to the amount of information which they convey, would be desirable. The first assumption is that each attribute is expressed as a scale on which each OTU can be described by a single unique position, i.e. the value of the OTU with respect to that attribute. Secondly, each observed attribute value is an estimate of the true position of the OTU on that scale, and has some degree of uncertainty associated with it, whether this uncertainty derives from technical, population or other irregularities. Thirdly, over a given scale, at a given confidence level, the confidence interval in scale units is con-





stant, i.e. independent of the attribute value. Fourthly, for a given confidence level, the relative information content for classification of any scale is the  $\log_2$  (number of classes + 1), these classes each being one confidence interval wide, and existing between the maximum and minimum observed values over the included OTU's. Fifthly, when only discrete classes are available for some attributes, these classes are assumed to be separated by one confidence interval at the 95% level. Thus the relative weight of such a scale is the log of the number of classes. For a continuous scale, where attribute values represent the means of small numbers of values, the average, over all the OTU's of the ranges about the means will be close to 4 standard deviations, and thus the average range can be taken as a 95% confidence interval. Where a large number of measurements have been made to determine each attribute value, standard deviations can be calculated, and their mean used to calculate a 95% confidence interval. Next, when the states of an attribute are classes which do not form a linear scale, it is convenient to assume that each class is one 95% confidence interval from every other class, and to weight a match in state between two OTU's as the log of the number of classes. A mismatch being given a weight of 1.0. Next, the position of an OTU on an attribute scale should not be dependent on its position on any other single scale. Finally, observed correlations between the states of OTU's on different attributes are not taken to be evidence for redundancy in the above sense and are not used for weighting.

The relative difference ( $d_{xy}$ ) between the states (V) of the  $x^{\text{th}}$  and  $y^{\text{th}}$  OTU's on a single attribute is taken to be quantified by

$$d_{xy} = V_x - V_y / (V_{\text{max}} - V_{\text{min}}).$$



i.e. The difference between the observed values divided by the observed range over all the OTU's. The relative difference between the  $x^{\text{th}}$  and  $y^{\text{th}}$  OTU's based on a number of attributes, is taken to be the weighted (w) arithmetic average of their relative distances on each attribute.

Partition of the OTU's into clusters defined within hyperspace is achieved by determining the proximities between pairs of OTU's, and rank ordering these, starting with the closest pair. The range of remaining distances is determined, and this distance divided by ten is designated CUT, which is added to the smallest distance in the middle 90% of the rank-ordered list of proximities, to give a distance QUIT. Pairs which are closer than the distance designated QUIT are considered to form the nucleus of the cluster. The closest point to any already in the cluster is then added to the cluster unless a) its distance from the closest OTU is greater than QUIT and its distance minus the average of the best and worst of such previous distances is greater than CUT, or b), its average distance from all previously admitted points is greater than QUIT and the average for this point minus the average for the last previously admitted point is greater than CUT, or c) a ratio criterion which prevents scattered points from bridging large elongated clusters is not met, or d) the closest point is already a member of a previously formed cluster. OTU's which are farther than QUIT from any other OTU's and which were excluded from diffuse clusters by the above criteria are considered to be single member clusters.

The expression of multidimensional relationships graphically and therefore in two dimensions, necessarily leads to distortions of distance relations between the items. The TAXMAP procedure maintains un-





distorted the maximum intracluster distance and at least two of the nearest intercluster discontinuities, distorts some relations as specified in the map, and omits others. This method preserves what are assumed to be the most important relations in the classification.



#### IV

#### MATERIALS AND METHODS



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### Organisms.

Strains designated NCTC were obtained from the National Collection of Type Cultures, Colindale, London, England.

Strains designated NCIB were obtained from the National Collection of Industrial Bacteria, Aberdeen, Scotland.

Strains designated CCM were obtained from the Czechoslovak Collection of Microorganisms, J.E. Purkyne University, Brno, Czechoslovakia.

The organisms used in this study fall into four distinct groups. The first of these groups comprises strains of Gram-negative, agar-putrefying bacilli, some of which have been called Bacteroides corrodens. A variety of corroding bacilli have been reported, (Eiken, 1958; Khairat, 1967; Henrikson, 1969), but the strains described differ somewhat in their cultural characteristics. Strains which conform to Eiken's description in biochemical and morphological characteristics are usually facultative anaerobes, and appear to be related antigenically. Strains designated B. corrodens by Khairat are strict anaerobes. The organisms give negative results in many of the routine diagnostic test and are therefore very difficult to classify by conventional methods on the basis of negative results.

#### I. Facultative strains.

- 1) B. corrodens NCTC 10596. Henrikson's type strain 333/54-55.
- 2) B. corrodens A40/68, obtained from the National Collection of Type Cultures at Colindale.





- 3) B-916, obtained from Dr. S.M. Finegold, Veterans Administration Centre, Los Angeles, Calif.
- 4) 53 P was a strain isolated at the University of Alberta Hospital (UAH) Edmonton, being very similar in its characteristics to NCTC 10596.
- 5) 53 W was a non-pitting variant of 53 P.
- 6) R27-70 was isolated at UAH from a post-appendectomy infection.
- 7) S-209 was isolated from sputum at UAH.

## II. Anaerobic strains.

- 8) EDMH-1, strain Misericordia, was isolated from amniotic fluid at the Misericordia Hospital, Edmonton.
- 9) UAH-1, strain Lane (4282) was isolated from a face lesion at UAH.
- 10) B-912, was a strain tentatively designated B. *corrodens*, and obtained from Dr. S.M. Finegold.
- 11) Strain 3936, was isolated from sputum at UAH.
- 12) Strain 4482, was isolated from a neck lesion at UAH.

For purposes of comparison, biochemical tests were done on these organisms, and on the organisms in Group II, and the results were subjected to computer analysis.

The second group of organisms comprises strains and species other than B. *corrodens*, which are Gram-negative, anaerobic or facultatively anaerobic, and non-sporulating. Members of the genera Bacteroides, Vibrio (species placed by Sebald and Véron, (1963) into a new genus, Campylobacter), Fusobacterium and Haemophilus were studied.



- 1) Bacteroides fragilis, NCTC 9343.
- 2) Bacteroides oralis, a strain obtained from Miss E. Bergen, University of Alberta.
- 3) Bacteroides melaninogenicus, a strain isolated at UAH.
- 4) Vibrio sputorum JV 5, provided by Dr. W.J. Loesche, School of Dentistry, University of Michigan.
- 5) V. sputorum ER33, also provided by Dr. Loesche.
- 6) Vibrio bubulius, NCTC 10355.
- 7) Vibrio foetus, NCTC 10354.
- 8) V. foetus, NCTC 5850.
- 9) V. bubulius strain Waterloo, IP 53103 (Institut Pasteur, Paris).
- 10) Haemophilus aphrophilus, NCTC 5886.
- 11) A recently isolated strain of Haemophilus influenzae, 20308.
- 12) A recently isolated strain of Fusobacterium, from UAH.

The third group of organisms studied includes members of the family Micrococcaceae. Species and strains from this family were subjected to computer analysis by previous workers in this laboratory (Sihota, 1969), who found that on the basis of the biochemical tests employed, cytochrome components, and results of gas chromatography, certain strains labelled as Micrococcus species showed closer similarity with Staphylococcus species than with Micrococcus species. The base ratios of these strains were determined, together with those of other members of the family.

#### I. Organisms not placed in clusters at the first level of res-





olution in the computer analysis.

- 1) Micrococcus tetragenus.
- 2) Micrococcus halophilus.
- 3) Micrococcus cyaneus NCIB 9148.
- 4) M. cyaneus CCM 856.
- 5) Micrococcus freudenreichii NCIB 2699.
- 6) Micrococcus aurantiacus NCIB 8609.
- 7) Micrococcus candidus NCIB 8610.
- 8) Micrococcus roseus NCIB 8175.
- 9) M. roseus CCM 706 (Sarcina erythromyxa ATCC 8944).
- 10) Micrococcus varians CCM 884 (Staphylococcus lactis NCTC).
- 11) Micrococcus conglomeratus NCIB 2677.

II. Organisms placed in clusters at the first level of resolution in the computer analysis.

- 12) Micrococcus luteus CCM 852 (Micrococcus species LA 9.1) which appeared to be a 'typical' M. luteus.
- 13) Micrococcus violagabriellae NCTC 9865. This strain was not included in the computer analysis, but a strain designated M. violagabriellae Parent was included in a cluster with Staphylococcus albus 12731.

III. Organisms not included in the previous computer analysis but shown by Rosypal, Rosypalová and Hořejš<sup>✓</sup> (1966) to have intermediate GC contents, typical of neither the Staphylococcus group, nor the Micrococcus group.

- 14) Micrococcus aquivivus CCM 316 (Pianococcus citreus).
- 15) Micrococcus varians CCM 250 (Sarcina oliva).



The fourth group of organisms studied includes members of the genus Pasteurella. Many strains of P. multocida had been subjected to computer analysis by previous workers in this department (Goodman, 1964), and the clusters obtained were compared with the serological types of these organisms. Organisms in group I on cluster analysis were all found to belong to serotype A; group II comprised organisms which were all of serotype D, and groups III and IV were not typeable. Strains of other species of Pasteurella were also examined.

I. P. multocida.

Group I (A).

- 1) 10456-56 isolated from human sputum (3).
- 2) 5568-61       "       "       "       "       (37).
- 3) M4631-60     "       "       rabbit lung (61).

Group II (D).

- 4) 675-59       "       "       human sputum (15).
- 5) 10955-55     "       "       "       CSF (1).
- 6) 11085-55     "       "       "       "       (2).

Group III (NT).

- 7) 5228-58       "       "       cat bite (9).
- 8) MU3004-58    "       "       human peritonitis (6).
- 9) M6395-58     "       "       dog bite (7).
- 10) MU1109-61   "       "       "       "       (32).
- 11) M2373-62    "       "       cat gums (62).

Group IV (NT).

- 12) M191-60     "       "       dog bite (25).
- 13) MU2920-59   "       "       human thigh abscess.



Type C.

14) P. multocida type C.

II. Other Pasteurella species.

15) Pasteurella pseudotuberculosis, stock culture, Provincial Laboratory.

16) Pasteurella ureae MU 7572 isolated from human sputum (69).

17) P. ureae MU5105-60 isolated from human sputum (68).

18) Pasteurella pneumotropica M3913-66 isolated from mouse liver.

19) Pasteurella haemolytica M1583-62 isolated from a goat (65).

20) An organism tentatively classified as Pasteurella pfaffii SC 1-64.

Included for purposes of comparison with the Pasteurella species were certain other Gram-negative organisms;

1) Unclassified Gram-negative rod MU14249-64, isolated from the human uterine cavity. This organism was pathogenic to mice and behaved biochemically in a way similar to P. multocida.

2) Unclassified Gram-negative rod MU7120-69, isolated from a human foot. The organism was non-fermentative, oxidase positive, and catalase positive.

3) A strain of Proteus vulgaris isolated from urine.

4) A strain of Klebsiella, U113-70, isolated from urine.

5) A strain of Enterobacter, #62.

6) A strain of E. coli B.





### Maintenance.

Cultures were maintained on medium containing 2.5% (w/v) nutrient agar broth #2 (Oxoid), 1.5% (w/v) Bactoagar (Difco), and 5% (w/v) defibrinated sheeps' blood, in distilled water. The organisms were subcultured weekly, with the exception of strains of the genus Vibrio which were subcultured every two days. Incubation was at 37°C for 24 or 48 hours, in a suitable atmosphere.

The gas phase conditions routinely used were:

- i) Aerobic for strains of the genera Pasteurella, Micrococcus, Staphylococcus and for members of the family Enterobacteriaceae.
- ii) Aerobic + 5-10% CO<sub>2</sub>, the gas flow being regulated by National flowmeters into cabinets of approx. 1 cu.ft. capacity, for facultatively anaerobic strains of B. corrodens, and for H. aphrophilus and H. influenzae.
- iii) Anaerobic in Baird and Tatlock cold catalyst jars gassed with 10% CO<sub>2</sub> + balance hydrogen, for anaerobic strains of B. corrodens and other species of Bacteroides, and for the strain of Fusobacterium.
- iv) Anaerobic in Baird and Tatlock jars with catalyst removed, and gassed with a mixture of N<sub>2</sub>:H<sub>2</sub>:CO<sub>2</sub> (80:10:10), for members of the genus Vibrio.

### Estimation of the GC content of bacterial DNA.

- 1) Isolation of DNA.



The procedure used is that of Marmur and Doty (1962), with slight modifications.

Sufficient blood agar plates to yield approximately 2g. wet weight of cells were inoculated and incubated in a suitable atmosphere for 48 hours at 37°C. Growth was scraped from the surface of the plates and the organisms suspended in 50 ml. saline-EDTA (0.15 M NaCl + 0.1 M ethylenediaminetetra-acetate, tetrasodium salt), pH 8.0. The cells were collected by centrifugation in a Servall RC2 centrifuge equipped with an SS-1 rotor, and the pellets were resuspended in 25 ml. saline-EDTA, using a vortex (Super-mixer, Lab-Line Instruments, Inc.).

The suspension was transferred to a 125 ml. ground-glass stoppered flask. To suspensions of Gram-positive organisms, lysozyme (Grade I, 3x recrystallized, Sigma Chemical Company) was added to a final concentration of 10  $\mu\text{g./ml.}$ , and incubated at 37°C for up to 60 minutes. 2 ml. sodium lauryl sulphate (24% saturated solution in water) was added to 25 ml. of suspensions of Gram-negative organisms and to the lysozyme treated suspensions of Gram-positive cells, and the mixture incubated at 60°C in a waterbath (Precision Scientific Co.) for 10 minutes. Lysis of the cells was indicated by an increase in viscosity and a clearing of the suspension. The lysate was cooled to room temperature, and 7 ml. 5 M sodium perchlorate was added to give a final concentration of 1 M perchlorate, which served to dissociate protein from the DNA. The whole mixture was shaken with an equal volume of chloroform-isoamyl alcohol (24:1) on a Griffin Flask Shaker (Griffin and George, England), for 30 minutes to deproteinize the DNA released on lysis. The mixture was then centrifuged for 5 minutes at 10,000 r.p.m.





in the RC2, and separated into three layers. The DNA was contained in the upper aqueous layer which was then pipetted off into a 100 ml. graduated cylinder, and ethyl alcohol (95%), was layered carefully on top of the aqueous phase, to approx. 2x its volume. DNA was precipitated by gentle stirring at the interface of the two layers with a glass rod. Thread-like fibres of DNA usually wound around the end of the rod. If the DNA failed to spool, the precipitate was granular and could be collected by centrifugation in the Servall for 20 minutes at 13,000 r.p.m. The precipitate was then dispersed in 5 ml. dilute saline citrate ( $0.015 \text{ M NaCl} + 0.0015 \text{ M trisodium citrate}$ , pH  $7.0 \pm 0.2$ ) in a 50 ml. ground-glass stoppered flask. 0.5 ml. concentrated saline citrate ( $1.5 \text{ M NaCl} + 0.15 \text{ M trisodium citrate}$ ) was added to bring the buffer's ionic strength to that of standard saline citrate. RNase (Bovine pancrease, 5x recrystallized Sigma Chemical Company) in a stock solution of 0.2% (w/v) in  $0.15 \text{ M NaCl}$ , pH 5, which had been heated at  $80^{\circ}\text{C}$  for 10 minutes to inactivate any DNase present, was added to the mixture in a final concentration of  $50 \mu\text{g./ml.}$ , and incubated at  $37^{\circ}\text{C}$  for 30 minutes. An equal volume of chloroform-isoamyl alcohol was then added and the mixture shaken again for 15 minutes. The emulsion was then centrifuged at 10,000 r.p.m. for 5 minutes, the aqueous layer removed, and the DNA precipitated as before. Subsequent deproteinizations were performed if necessary. If sufficient DNA was obtained, the precipitate was dissolved in 9 ml. dilute saline citrate, and 1 ml. acetate-EDTA ( $3.0 \text{ M sodium acetate} + 0.001 \text{ M EDTA}$ , pH 7.0) added with rapid stirring. 0.54 volumes of isopropanol was added dropwise to this mixture with continuous stirring. The DNA precipitated could



be wound around a glass rod, and was then washed with 70%, 80%, 90% and 95% ethanol. This precipitate or that resulting from the final ethanol precipitation, was dispersed in a minimum volume of standard saline citrate (SSC, 0.15 M NaCl + 0.015 M trisodium citrate, pH 7.0  $\pm$  0.2). In some instances the final precipitate was divided into four portions, one of which was dissolved in each of SSC, SSC diluted 1/2, 1/5, and 1/10 with distilled water.

## 2) Determination of the melting temperature of DNA.

Melting temperatures were determined using a Beckman DB-G spectrophotometer, equipped with a heating cell, and a Beckman  $T_m$  analyser, and the absorbances were recorded on a Beckman 10" recorder. The  $T_m$  analyser was programmed to heat from 25°C through 85°C to a final temperature of 110°C, in a period of 100 minutes. The "percentage scan" registered on the  $T_m$  analyser and on the trace on the recorder was correlated in terms of °C by calibration using a thermistor probe (Beckman Instruments Ltd.). The probe was placed inside the sample cuvette within the heating chamber in the spectrophotometer, and SSC buffer put in both cuvettes. The  $T_m$  analyser was then allowed to equilibrate for 10 minutes, to its starting temperature of 25°C, and the programme then switched in. Temperature readings on the thermistor were taken for approx. every 5% of the scan. These values of temperature and % scan were plotted and the resultant straight line graph was used to convert percentage scan, as recorded, into °C (Fig. 1).

For determination of melting temperatures, the spectrophotometer was blanked using SSC (or whichever dilution of SSC the sample of DNA



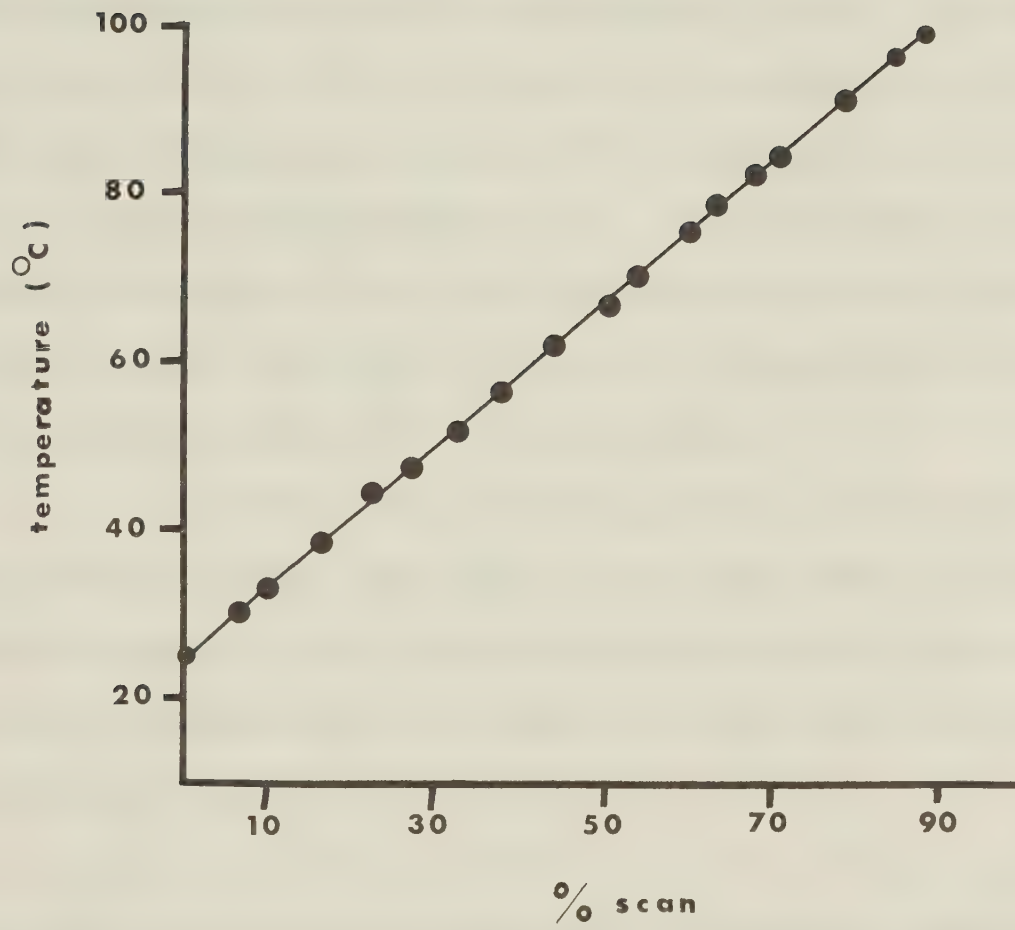




FIGURE 1

Plot of temperature ( $^{\circ}\text{C}$ ) with % scan for Standard Saline Citrate (SSC)  
in a Beckman DB-G spectrophotometer, with  $T_m$  analyser.

FIGURE 1





was dispersed in), in quartz cuvettes, and a base line was scanned from 310 nm. to 220 nm. The sample cuvette was then filled with the DNA preparation, and this was diluted with the appropriate buffer until an absorbance of approx. 0.5 was obtained. This preparation was then scanned from 310 nm. to 220 nm., and the scan recorded at 5 inches/minute. Good preparations of DNA gave maximum absorbance at 255-260 nm. with no indication of a peak at 280 nm. (the maximum absorbance of protein). The  $T_m$  analyser was then allowed to equilibrate to its starting temperature of 25°C, and the recording rate was adjusted to 0.1 inches/minute. The spectrophotometer was set at a wavelength of 260 nm. After the 10 minute equilibration period, the preset heating programme was switched in. The  $T_m$  was determined in terms of the percentage scan value at the midpoint (50%) of the hyperchromic shift. This was converted to °C by means of the graph of °C/ % scan previously described. The GC content of the DNA was then calculated from the  $T_m$  value, using the relationship derived by Owen, Hill and Lapage (1969)

$$\theta GC = [\tan (70.077 + 3.32 M_g)] (T_m - 175.95) + 260.34.$$

All calculations were done using a Monroe Epic 3000 calculator. In the first instance,  $T_m$  had been converted to GC% using a second graph of °C / %GC as determined by other methods, but this was found to give inaccurate and inconsistent results.

### 3) Effect of sodium ion concentration on the melting temperature of DNA.

The sodium ion content of SSC was estimated by flame photometry, and dilutions of 1/2, 1/5, and 1/10 of SSC in distilled water were prepared. DNA was extracted and deproteinized as before, and the final





precipitate was divided into four portions, one of which was dispersed in each dilution of SSC, and one in undiluted SSC. These four preparations were used to determine the  $T_m$  as before. The melting temperature of DNA from organisms representative of a wide range of GC contents was determined in each of the four concentrations of sodium ion. After these  $T_m$  values had been shown to be consistent and reliable in all four dilutions of SSC by substitution in the equation of Owen, Hill and Lapage, the diluted SSC was used routinely in the estimation of  $T_m$  in those organisms which have a high G + C ratio, e.g. members of the genus Micrococcus.

#### Biochemical Tests used with some Organisms of Groups I and II.

The basal medium used consisted of 2.5% (w/v) powdered nutrient broth #2 (Oxoid), 1.5% (w/v) Bactoagar (Difco), and 0.1% (w/v)  $\text{KNO}_3$  in distilled water. This was sterilized by autoclaving for 15 minutes at  $121^\circ\text{C}$ . Cysteine-HCl, dissolved in HCl by boiling, was added to the sterilized medium in a final concentration of 0.005% (w/v). Haemin, (prepared as a stock solution by dissolving recrystallized haemin (Eastman Organic Chemicals) in 0.5%  $\text{Na}_2\text{CO}_3 \cdot \text{H}_2\text{O}$  to a concentration of 5,000  $\mu\text{g./ml.}$ ) was sterilized by millipore filtration and added to the sterile medium in a final concentration of 25  $\mu\text{g./ml.}$

i) Gas phase conditions. Suspensions of each organism were made in nutrient broth, and the absorbance of the suspension was adjusted to 0.5 as measured on a Bausch and Lomb Spectronic 20 spectrophotometer. This suspension was then diluted 1/100 with nutrient broth, and inoculated onto plates of basal medium using a Ridgeway Watt multipoint inoculator



(Ridgeway Watt, Epsom, England). Inoculated plates were incubated under five gas phase conditions.

i) Air.

ii) Air + 5-10% CO<sub>2</sub>, the gas mixture being regulated by National flowmeters, and fed into cabinets of approx. 1 cu.ft. capacity.

iii) In Baird and Tatlock cold catalyst jars gassed with a mixture of 90% H<sub>2</sub>: 10% CO<sub>2</sub>.

iv) In Baird and Tatlock jars, without catalysts, gassed with a mixture of 1% O<sub>2</sub>: 10% CO<sub>2</sub>: hydrogen balance.

v) In Baird and Tatlock jars without catalysts, gassed with a mixture of 5% O<sub>2</sub>: 10% CO<sub>2</sub>: nitrogen balance.

ii) Oxidase reaction. a) Filter paper was moistened with a 1% solution of dimethylparaphenylenediamine, and colonies transferred onto this with a platinum loop. Development of a strong red colour in 10 seconds indicated a positive reaction.

b) Filter paper was moistened with a 1% solution of tetramethylparaphenylenediamine and colonies transferred onto this with a platinum loop. Development of a strong purple colour within 10 seconds indicated a positive reaction.

iii) Catalase reaction. Colonies of bacteria were removed from agar plates with a wire (pretested for inertness), and immersed in 2 ml. of 3% H<sub>2</sub>O<sub>2</sub> in a small tube. Immediate production of bubbles indicated a positive reaction.

iv) Nitrate reduction. Cook's method (1950) was used. Plates of 5% blood agar containing 0.1% KNO<sub>3</sub> were stabbed with a loopful of the



organism, and incubated anaerobically for 24 hours at 37°C. After incubation the plates were left at room temperature for 30 minutes and then read. A brown zone appears around the stab of nitrate-reducing organisms.

v) Starch hydrolysis. Soluble starch (Difco) was added to melted basal medium at a concentration of 0.1% (w/v), and the medium autoclaved for 15 minutes at 115°C, cooled, and poured into plastic petri dishes. Plates were inoculated as for gas phase experiments, and incubated at 37°C for 5 days in each of three atmospheres,

i) Air + 5-10% CO<sub>2</sub> (q.v.).

ii) Anaerobically in Baird and Tatlock cold catalyst jars gassed with 90% H<sub>2</sub>: 10% CO<sub>2</sub>.

iii) Anaerobically in Baird and Tatlock jars without catalysts gassed with 80% N<sub>2</sub>: 10% CO<sub>2</sub>: 10% H<sub>2</sub>.

After incubation, Lugol's iodine, diluted 1/5 was poured onto the plates; clear zones around colonies indicated hydrolysis of starch.

vi) Gelatin hydrolysis. Gelatin (Difco) was added to melted basal medium at a final concentration of 0.4 (w/v). The medium was cooled and poured into plastic petri dishes, and plates were inoculated and incubated as described for starch hydrolysis. After incubation plates were flooded with acid mercuric chloride reagent, and clear zones around the colonies indicated hydrolysis of gelatin.

vii) Casein hydrolysis. Skim milk (Difco) was dissolved in distilled water at 10% (w/v) concentration. 250 ml. of this solution was added to an equal volume of double strength basal medium which had been autoclaved and cooled. Plates were poured, inoculated and incubated as before. After 7 days incubation, clear zones around the colonies





were noted, and the plates flooded with acid mercuric chloride reagent to distinguish between true proteolysis and clearing due to solubilization of the milk proteins by alkaline products of metabolism.

viii) Tyrosine hydrolysis. Tyrosine (insoluble) was added to basal medium as a final concentration of 1% (w/v). The medium was autoclaved, cooled and poured as before, and plates were inoculated as before, and incubated for 10 days. Clearing around the colonies indicated hydrolysis of tyrosine.

ix) Malonate utilization and phenylalanine deaminase activity. Powdered malonate broth (Difco) 0.8% (w/v), and phenylalanine agar (Difco) 2.3% (w/v) were dissolved in distilled water, and haemin added to a final concentration of 25  $\mu\text{g./ml.}$  The medium was autoclaved, cooled and poured. Plates were inoculated as before and incubated for up to 5 days. Development of a blue colour indicated malonate utilization and the plates were then flooded with N HCl, and then with 10%  $\text{FeCl}_3$  to indicate phenylalanine deaminase activity.

x) Citrate utilization. Haemin was added to Simmon's citrate agar at a final concentration of 25  $\mu\text{g./ml.}$ , and the medium autoclaved, cooled and poured as before. Plates were inoculated and incubated for 10 days. Development of a blue colour indicated utilization of citrate.

xi) DNase activity. Plates of DNase agar (BBL) (4.2% w/v) containing 25  $\mu\text{g./ml.}$  of haemin were poured, inoculated and incubated as before. After 3 days incubation, they were flooded with N HCl, and examined for clear zones indicating the breakdown of DNA.

xii) Resistance to sodium azide.  $\text{NaN}_3$  was added to basal medium in a final concentration of 0.2% (w/v). Plates were poured, inoculated and



incubated as before, and examined for growth after 3 days.

xiii) Bile tolerance. Bile was added to basal medium at final concentrations of 10% and 40%. Plates were poured, inoculated and incubated as before, and examined after 3 days for growth.

xiv) Indole production. a) NCTC micromethod (Cowan and Steele, 1965). 0.06 ml. tryptophan (0.1%), 0.04 ml. 0.025 M phosphate buffer (50 ml. 0.025 M  $\text{Na}_2\text{HPO}_4$  + 50 ml. 0.025 M  $\text{KH}_2\text{PO}_4$ ) pH 6.8, and 0.04 ml. of a heavy suspension of organisms were incubated together in small tubes at 37°C for 1 hour. 0.06 ml. of Kovac's reagent was then added, and a pink colour indicated a positive reaction.

b) Filter paper strips soaked in oxalic acid, were placed in the lids of heavily inoculated blood agar plates, for the duration of incubation.

xv) Hydrogen sulphide production. Strips of filter paper soaked in 10% lead acetate solution, were placed in the lids of heavily inoculated blood plates and incubated for 24 hours.  $\text{H}_2\text{S}$  production was indicated by blackening of the strips.

Amino acid decarboxylases. NCTC micromethod.

xvi) 1) Arginine: 0.04 ml. of a 0.03 M solution of arginine (pH 5), 0.04 ml. of 0.0125 M phthalate buffer (50 ml. 0.05 M potassium hydrogen phthalate + 23.85 ml. 0.05 M NaOH made up to 200 ml. with distilled water, and adjusted to pH 5) + 2.5% (v/v) 1% ethanolic bromocresol purple, and 0.04 ml. of a heavy suspension of organisms were incubated together in small tubes at 37°C, and examined at 2, 4 and 24 hours for a change in colour of the indicator from yellow to purple, which would show the decarboxylation of arginine.





xvii) ii) Lysine: As for arginine, substituting a 0.03 M solution of lysine for arginine.

xviii) iii) Ornithine: As for arginine, substituting a 0.03 M solution of ornithine for arginine.

xix) Urease activity. Urease medium (Difco) was dissolved in distilled water by boiling, at a concentration of 2.4% (w/final volume), and the pH adjusted to 7.3. Urea, as a 40% solution was added to the cooled melted medium in a final concentration of 5% (v/v). Slants of this medium were inoculated, and incubated as before for 24 hours. A pink colour indicated urease activity.

xx) Aesculin hydrolysis: Slants of aesculin medium (aesculin 0.1% (w/v), ferric citrate 0.05% (w/v), heart infusion agar 40% (w/v) in distilled water, pH 7.0) were inoculated, and incubated at 37°C in a suitable atmosphere.

xxi) Starch-acid production. Serum water base (p. 109, Cowan and Steele, 1965), + 3% (w/v) soluble starch was inoculated with each organism, and incubated at 37°C for 2 days in suitable gas phase conditions. Development of a pink colour indicated production of acid from starch.

xxii) Motility. i) Motility was tested using the motility-indole medium described by Blazevic (1968). Organisms were inoculated with a straight wire to one half the depth of the medium, and were incubated for 2 days at 37°C in a suitable atmosphere.

ii) Motility was looked for in hanging drops and in wet preparations under cover slips. As these methods might be unsuitable for anaerobes, a special method was devised. Strips of aluminum 1.5 mm.



in thickness and 2.5 cm. wide were cut into 3.5 cm. lengths. A hole 1.25 cm. in diameter was drilled in the centre of each. These metal components were sterilized by flaming, and attached by paraffin wax to heat-sterilized slides. A small volume of melted cystine-haemin nutrient agar medium (see below) was run into the well formed by the drilled metal, to about two-thirds of the depth. After solidification had occurred the organism to be tested was inoculated at a point near the periphery, and the well was filled with nutrient broth. A cover slip was applied, and sealed at the edges with vaseline after excess fluid had been removed with filter paper. With anaerobes, transfer of organisms was carried out in a glove box gassed with oxygen-free nitrogen with 10% CO<sub>2</sub>. The slides were incubated and observed at intervals by means of xl6 phase contrast objective, an Optovar setting of 2 and xl2.5 wide field eyepieces. This gave an effective magnification of 400 diameters, sufficient resolution for initial scanning, and permitted the examination of large populations for the presence of occasional motile organisms. Higher power objectives were used to search for possible minute motile forms. This method was excellent for the detection of motile vibrios in which only a small proportion of the cells was flagellated.

Cultures were examined for motility 4 hours after inoculation, and at intervals thereafter up to 3 days. Incubation temperatures of 37°C, 30°C and 25°C were used.

#### Analysis of biochemical data.

The results of the biochemical tests in section 3 were tabulated and scored for computer analysis. The majority of tests were negative or



positive in their results, and as such were scored as neg = 0/ pos = 1. Results of growth in different gas phase conditions were weighted since five equally reliable states were logically possible. These states were scored on a scale from 0 to 4: growth in air = 4; growth in air + CO<sub>2</sub> = 3; growth in 5% O<sub>2</sub> = 2; growth in 1% O<sub>2</sub> = 1; growth only anaerobically = 0.

Similarly, since any organism which gave a positive oxidase test result with the dimethyl reagent also gave a positive result with the tetramethyl reagent, these characters were scored as: giving a positive result with both reagents = 2; giving a positive result only with the tetramethyl reagent = 1; negative result with both reagents = 0.

Tolerance to 10% and to 40% bile were similarly scored; tolerance to both 40% and 10% = 2; tolerance to 10% only = 1; not tolerant of 10% = 0.

In the case of the ornithine decarboxylase test, repetition of the test several times gave variable results. Therefore three alternative states were scored for: positive = 2; variable = 1; negative = 0, but the weighting of the character was so adjusted that this three state scale occupied only one 95% confidence interval, and was thus not given more weight than the other negative/positive tests.

In using the DNA base ratios in the information to be analysed, a weight was assigned to them which was relative to their information content in comparison with the other data. The range of values obtained for the GC content of each organism over 2 to 6 measurements was found, and the mean of these ranges for all the OTU's was calculated. This value was found to be 1% which was taken to be close to 4 stand-





ard deviations and was used as a 95% confidence interval. The GC % formed a continuous scale from 28.3% to 65.3%, which range was then divided by 1.0 (as the 95% confidence interval) to give the number of possible classes each one confidence interval wide, which number, plus 1 was used to weight the character. The relative information content of the character is the log to the base 2 of the (number of classes + 1).

The data to be analysed then were punched on cards. Necessary for the operation of the programme used are a title card, options card, a card with the unique labels for all the OTU's, an attribute specification card, and an attribute value card., (Carmichael and Sneath, 1969);. The programme used was the Fortran IV programme by Dr. J.W. Carmichael of this department. The data were analysed weighted and unweighted, and the relationships between the OTU's were shown in TAXMAPS.



## RESULTS





## RESULTS

### I. Effect of sodium ion concentration on $T_m$ .

Four concentrations of buffer, SSC ( $0.192 \text{ M Na}^+$ ), SSC/2, SSC/5 and SSC/10 were used to determine melting temperatures of five organisms; - anaerobic Gram-negative rods B912 and 3936; the facultative anaerobe B. *corrodens* NCTC 10596, strain 333/54-55; M. *cyaneus* CCM 856; M. *luteus* CCM 852. These organisms were selected to cover a broad range of GC contents, from 28% to 71% GC.

The results of these estimations are shown in Table 1. The variation in GC contents calculated from the  $T_m$  in different concentrations of sodium ion was no greater than the variation in % GC found in doing replicate experiments with the same buffer.

Plots of melting temperature with  $-\log \text{M} (\text{Na}^+)$  are shown for each of the five organisms in Fig. 2. As shown by Owen, Hill and Lapage (1969), a linear relationship can be demonstrated between ionic concentration and  $T_m$ , but some slight convergence occurs between the linear graphs of the different organisms. However, use of the equation derived by the previous workers,

$$\theta \text{ GC} = [\text{Tan } (70.077 + 3.32 \text{ M}_g)] (T_m - 175.95) + 260.34$$

was found to give accurate and reliable conversions of the  $T_m$  found in the different ionic strength buffers, to % GC.

The influence of ionic strength on the thermal transition of the DNA can also be clearly shown by plotting the absorbance at various temperatures calculated relative to the absorbance at  $25^\circ\text{C}$ , with



TABLE 1

RESULTS OF  $T_m$  AND GC DETERMINATIONS IN FOUR IONIC CONCENTRATIONS

| Organism            | $-\log M$ ( $Na^+$ ) | SSC    | % scan | $T_m$ °C | % GC | Mean % GC |
|---------------------|----------------------|--------|--------|----------|------|-----------|
| dilution            |                      |        |        |          |      |           |
| B-912               | 0.7167               | SSC    | 64.50  | 80.50    | 27.7 |           |
|                     | 1.0179               | SSC/2  | 60.00  | 75.50    | 27.1 |           |
|                     | 1.4216               | SSC/5  | 54.75  | 70.00    | 29.9 |           |
|                     | 1.7167               | SSC/10 | 50.50  | 65.25    | 29.5 | 28.5      |
| 3936                | 0.7167               | SSC    | 71.50  | 85.00    | 38.7 |           |
|                     | 1.0179               | SSC/2  | 65.50  | 80.25    | 38.1 |           |
|                     | 1.4216               | SSC/5  | 59.00  | 74.50    | 38.2 |           |
|                     | 1.7167               | SSC/10 | 52.50  | 69.75    | 38.9 | 38.5      |
| <u>B. corrodens</u> | 0.7167               | SSC    | 80.50  | 92.50    | 57.8 |           |
| NCTC 10596          | 1.0179               | SSC/2  | 75.50  | 88.75    | 57.9 |           |
| 333/54-55           | 1.4216               | SSC/5  | 69.00  | 83.00    | 57.7 |           |
|                     | 1.7167               | SSC/10 | 63.50  | 78.75    | 57.1 | 57.6      |
| <u>M. cyaneus</u>   | 0.7167               | SSC    | 86.00  | 97.00    | 67.9 |           |
| CCM 856             | 1.0179               | SSC/2  | 81.00  | 93.00    | 67.7 |           |
|                     | 1.4216               | SSC/5  | 74.25  | 87.50    | 67.5 |           |
|                     | 1.7167               | SSC/10 | 69.25  | 83.25    | 67.0 | 67.5      |



TABLE 1 cont'd

| Organism         | - log <u>M</u> (Na <sup>+</sup> ) | SSC      | % scan | T <sub>m</sub> °C | % GC | Mean % GC |
|------------------|-----------------------------------|----------|--------|-------------------|------|-----------|
|                  |                                   | dilution |        |                   |      |           |
| <u>M. luteus</u> | 0.7167                            | SSC      | 87.00  | 98.50             | 70.4 |           |
|                  | 1.0179                            | SSC/2    | 84.00  | 94.00             | 70.4 |           |
|                  | 1.4216                            | SSC/5    | 76.00  | 89.00             | 70.8 |           |
|                  | 1.7167                            | SSC/10   | 71.00  | 84.50             | 69.6 | 70.3      |





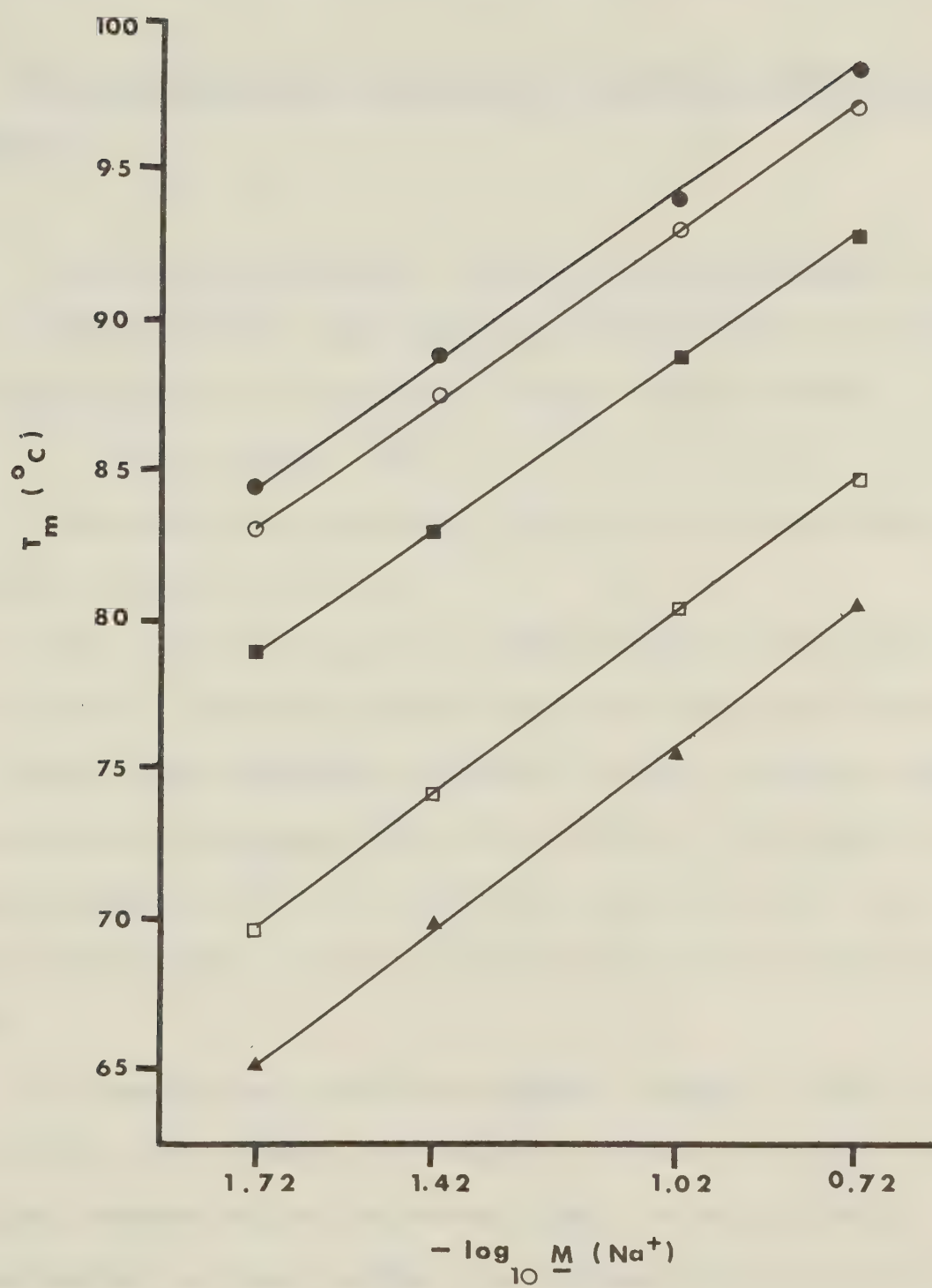


FIGURE 2

Plots of melting temperature ( $^{\circ}\text{C}$ ) with  $-\log \underline{M}$  ( $\text{Na}^+$ ) for:

|                                      |   |
|--------------------------------------|---|
| Anaerobic corroding bacillus B-912   | ▲ |
| Anaerobic corroding bacillus 3936    | □ |
| <u>B. corrodens</u> 10596 333 /54-55 | ■ |
| <u>M. cyaneus</u> CCM 856            | ● |
| <u>M. luteus</u> CCM 852             | ● |

FIGURE 2





temperature. This relationship found in the four buffers used is shown for M. cyaneus CCM 856 in Fig. 3.

The high degree of correlation found between estimations of %GC in the different buffers was considered sufficient to justify the use of dilute SSC in the routine determination of base ratios of Micrococci.

## II. Determination of the % GC content of strains resembling B. corrodens, (Group I).

The melting temperatures of DNA from strains included in Group I in the methods section, were determined in SSC, and the % GC contents were calculated from the equation of Marmur and Doty (1962)

$$\theta \text{ GC} = 2.44 T_m - 169.$$

The mean % GC was evaluated from two to six estimations, and the results are shown in Table 2.

Three distinct groups can be observed from the base ratios of these strains. The first comprises organisms which have 56.9%-57.9% GC, and includes the facultatively anaerobic strains, resembling Henrikson's type species, B. corrodens NCTC 10596, 333/54-55. The absorption spectrum and melting curve of DNA from this strain are shown in Fig. 4 as being representative of the facultatively anaerobic corroding bacilli used.

The second group comprises organisms having GC contents of 28.0%-29.7%, and includes four obligately anaerobic strains. The absorption spectrum and melting curve of DNA from strain EDMH-1 (Misericordia) are shown in Fig. 5 as representative of those of organ-







FIGURE 3

Plot of absorbance at 260 nm. at various temperature relative to absorbance at 260 nm. at 25°C, with temperature (°C) for M. cyaneus CCM 856 in four ionic concentrations.

|        |   |
|--------|---|
| SSC    | □ |
| SSC/2  | ■ |
| SSC/5  | ○ |
| SSC/10 | ● |

FIGURE 3

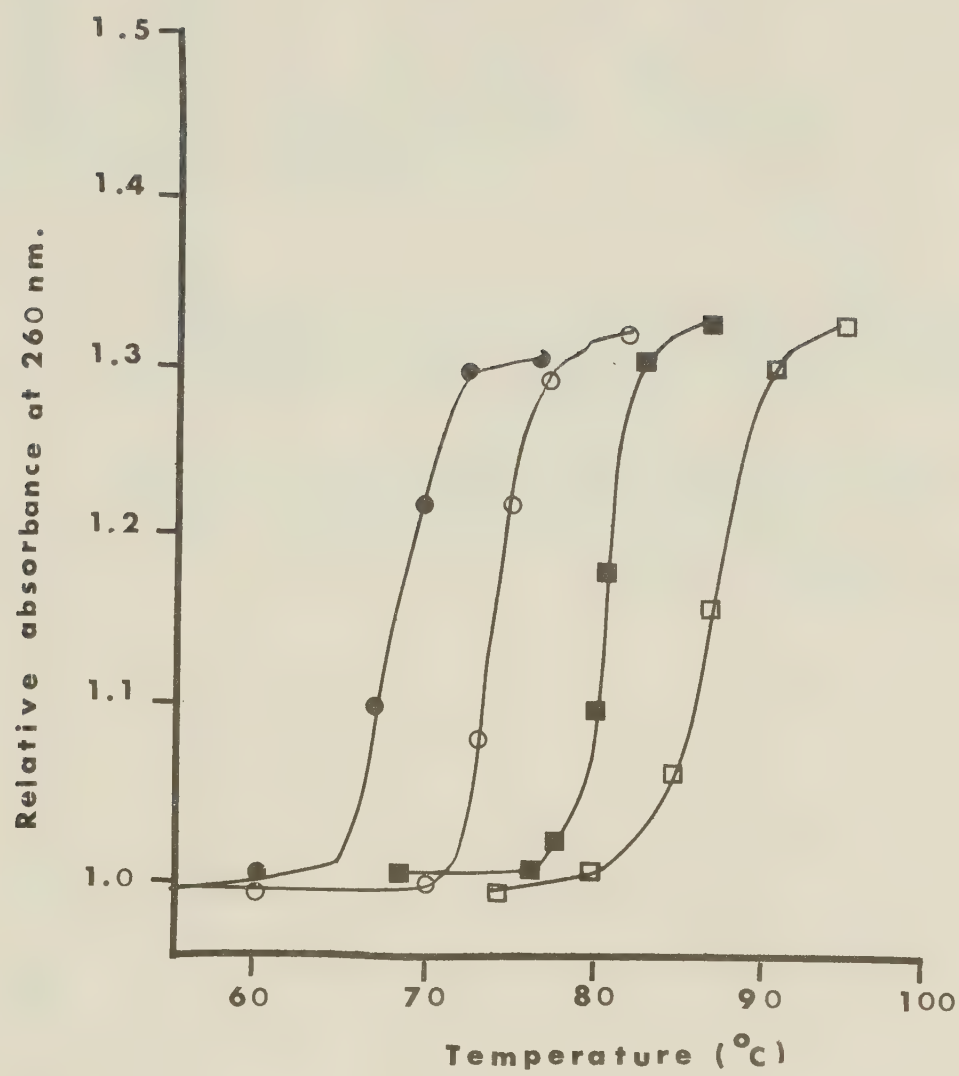




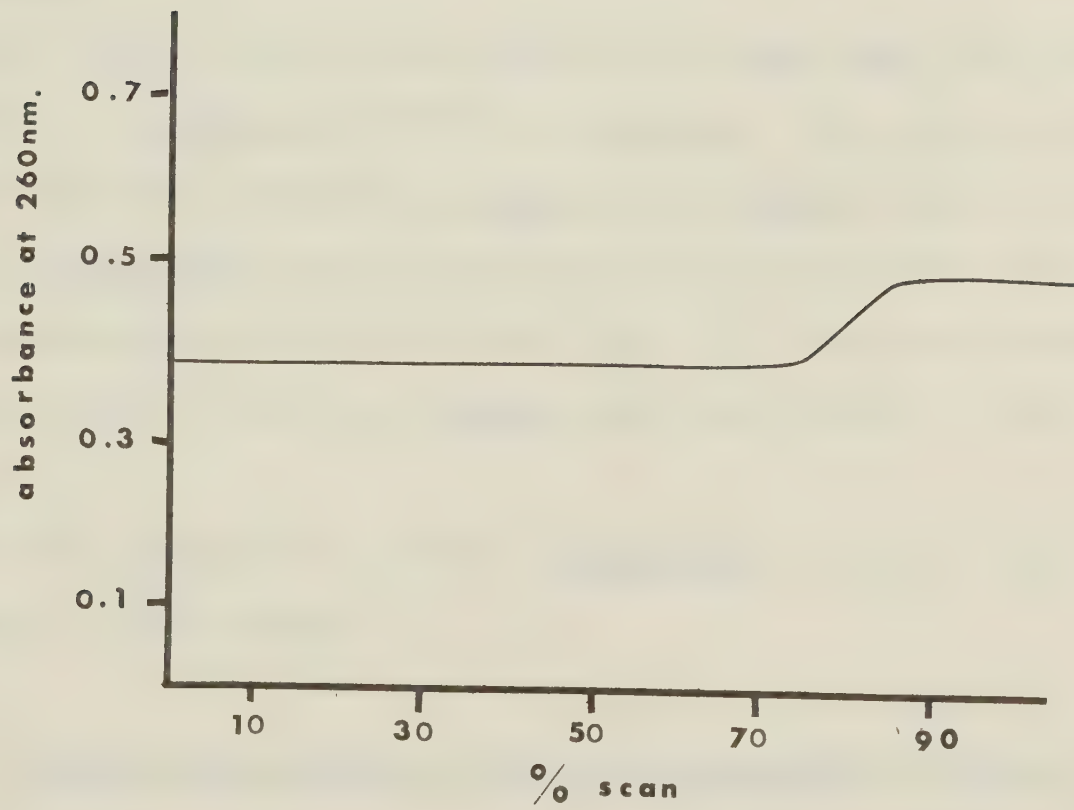
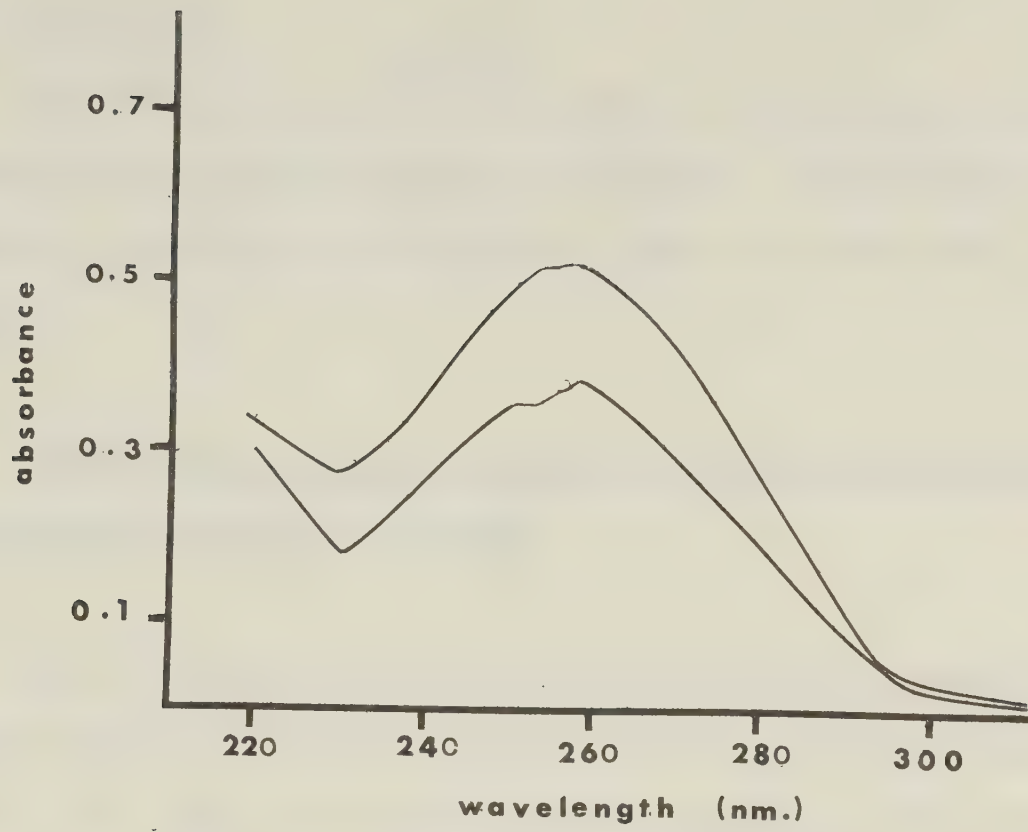


FIGURE 4

Ultraviolet absorption spectrum and melting curve at 260 nm.  
of DNA from B. *corrodens* NCTC 10596 333 / 54-55.



FIGURE 4





isms in this group.

The third group has only two members, the anaerobic strains 3936 and 4482, which show base ratios of 38%-39% GC. A representative absorption spectrum and melting curve of DNA from strain 4482 is shown in Fig. 6.

### III. Determination of the % GC content of some strains of Gram-negative bacilli which were not *B. corrodens*.

The GC contents of DNA from organisms included in Group II in the methods section were estimated in SSC, and the results are shown in Table 3. The values obtained for the non-corroding species of Bacteroides (41%-43% GC) are similar to the value previously obtained for B. insolitus.

The base ratios found for the species of the genus Vibrio vary from 46.9% for V. sputorum, to 29.5% for V. bubulus. The proposal by Sebald and Véron (1963) to include V. foetus and V. bubulus within a separate genus Campylobacter would preclude the incorporation within one genus of species with widely variant base ratios. The absorption spectrum and melting curve of DNA from V. bubulus (Waterloo) IP53103 is shown in Fig. 7.

% GC values obtained for species of Haemophilus are close to the values reported by other workers.

### IV. Computer-aided analysis of data from some members of Groups I and II.





FIGURE 5

Ultraviolet absorption spectrum and melting curve at 260 nm.  
of DNA from strain EDMH-1 (Misericordia).



FIGURE 5

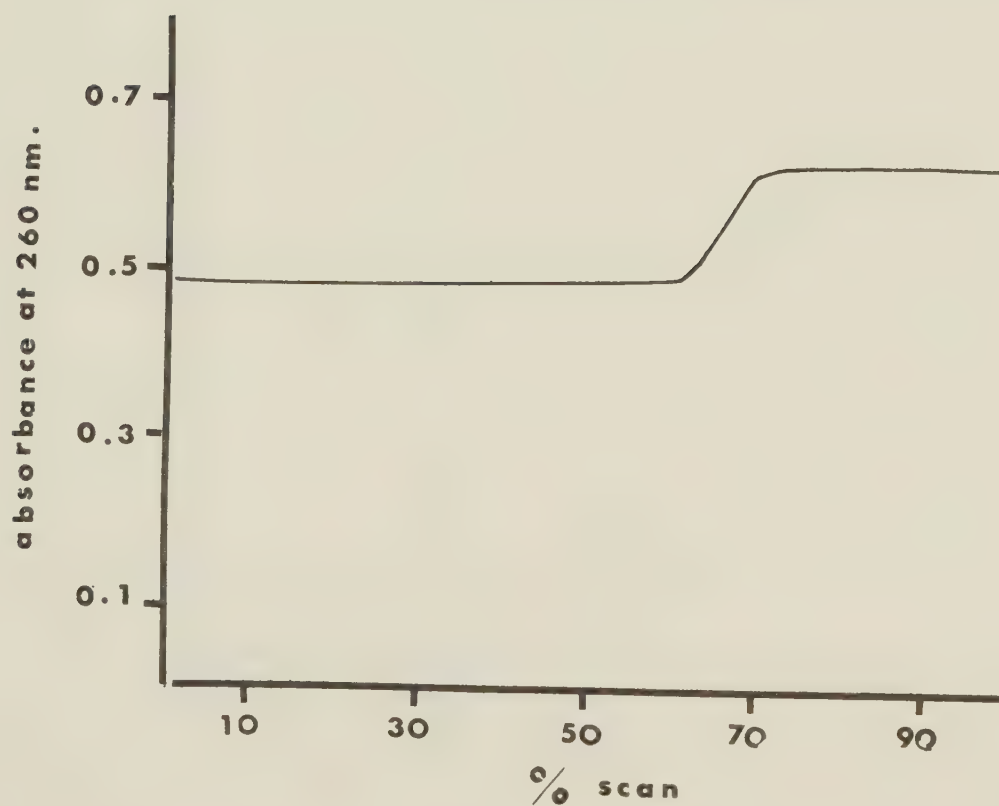
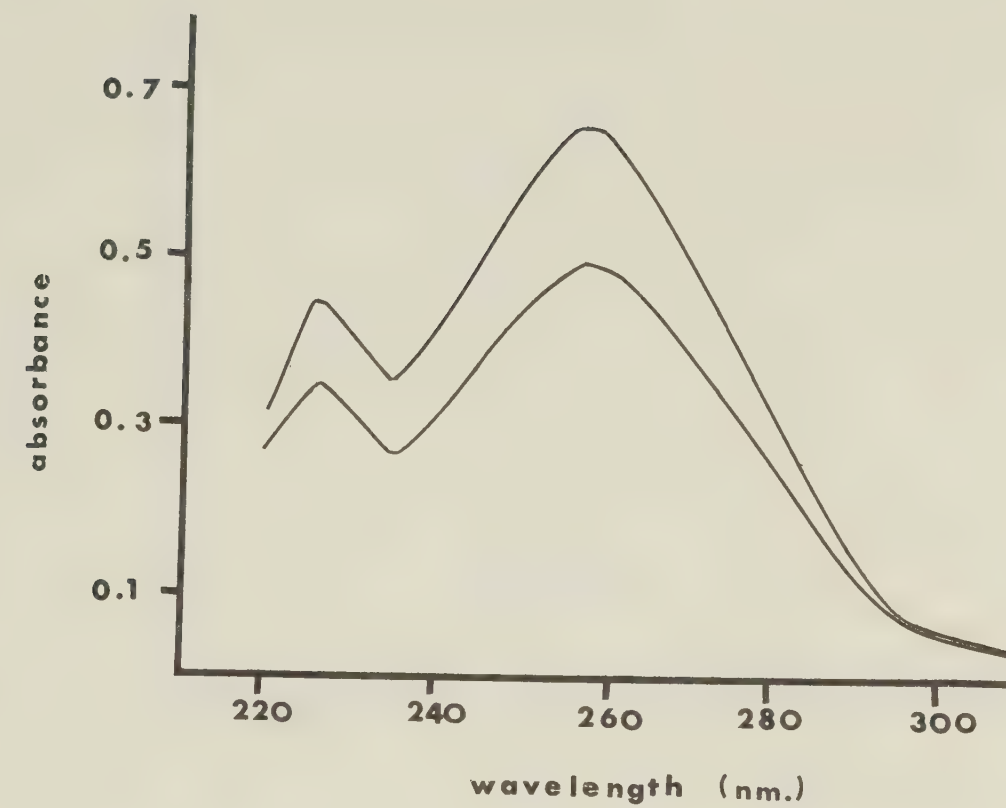






FIGURE 6

Ultraviolet absorption spectrum and melting curve at 260 nm.  
of DNA from strain 4482.

FIGURE 6

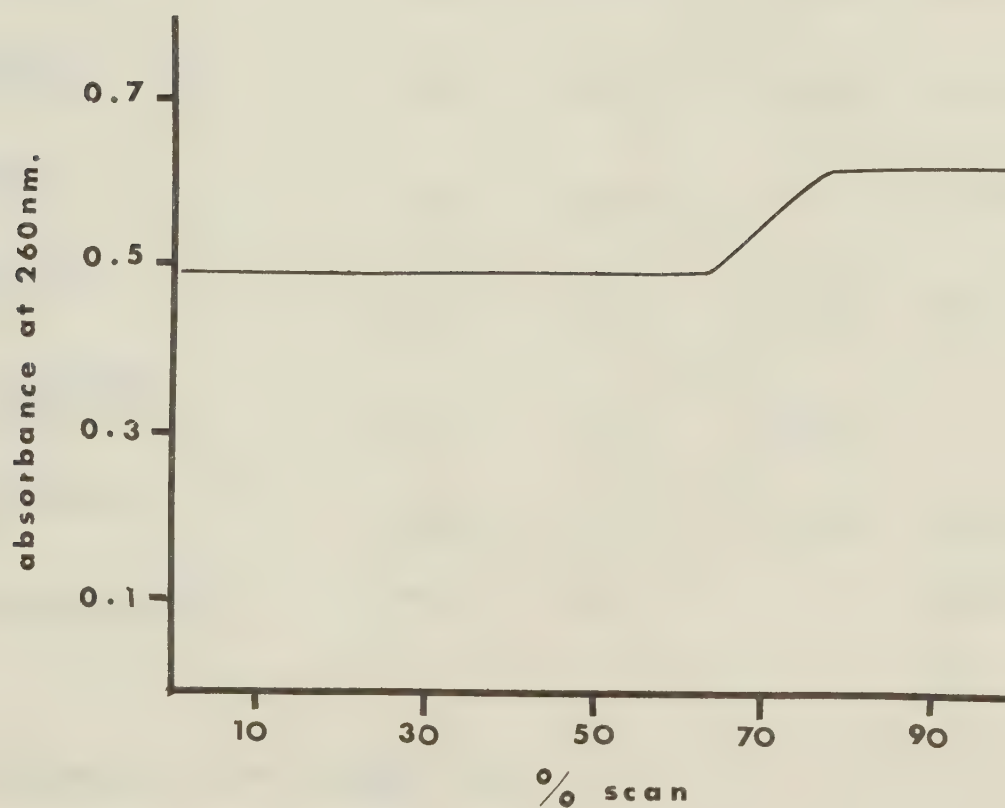
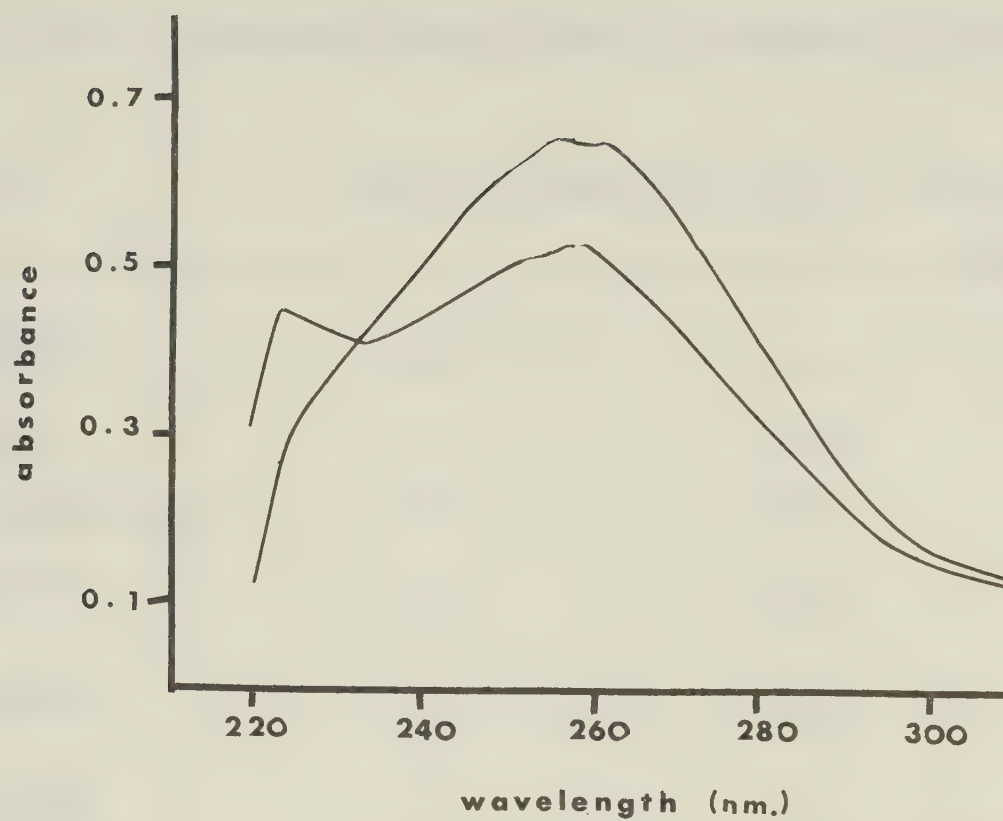






TABLE 3

BASE RATIOS OF GRAM-NEGATIVE RODS (GROUP II) DETERMINED IN SSC\*

| Organism                           | Mean GC% | Range % GC | Mean T <sub>m</sub> | Literature value | Ref.  |
|------------------------------------|----------|------------|---------------------|------------------|-------|
| <u>B. fragilis</u><br>NCTC 9343    | 42.2     | 1.7        | 86.50               | -                | -     |
| <u>B. oralis</u>                   | 43.0     | 0.5        | 87.00               | -                | -     |
| <u>B. melaninogenicus</u>          | 41.0     | 0.3        | 86.00               | -                | -     |
| <u>V. sputorum</u><br>JV 5         | 46.9     | 0.0        | 88.50               | -                | -     |
| <u>V. sputorum</u><br>ER 33        | 46.8     | 0.3        | 88.50               | -                | -     |
| <u>V. bubulus</u><br>NCTC 10355    | 29.9     | 0.1        | 81.50               | 29%-31%          | (101) |
| <u>V. foetus</u><br>NCTC 10354     | 32.3     | 0.0        | 82.50               | 33%-36%          | (101) |
| <u>V. foetus</u><br>NCTC 5850      | 32.0     | 0.5        | 82.25               | 33%-36%          | (101) |
| <u>V. bubulus</u><br>Waterloo      | 29.5     | 1.2        | 81.50               | 29.5%            | (101) |
| <u>H. aphrophilus</u><br>NCTC 5886 | 41.3     | 0.7        | 86.00               | -                | -     |
| <u>H. influenzae</u><br>20308      | 37.2     | 1.2        | 84.50               | 38%-40%          | (78)  |
| <u>Fusobacterium</u> sp.*          | 65.3     | 0.6        |                     | 31%-63%          | (58)  |

\*T<sub>m</sub> determined in SSC diluted 1/10.

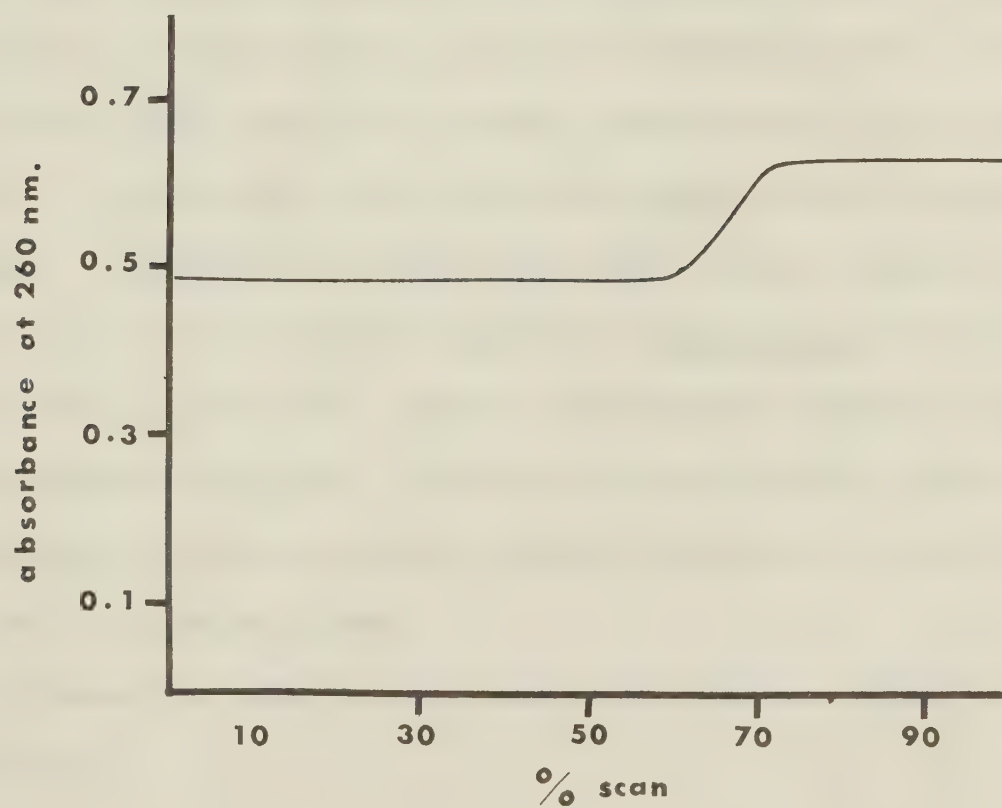
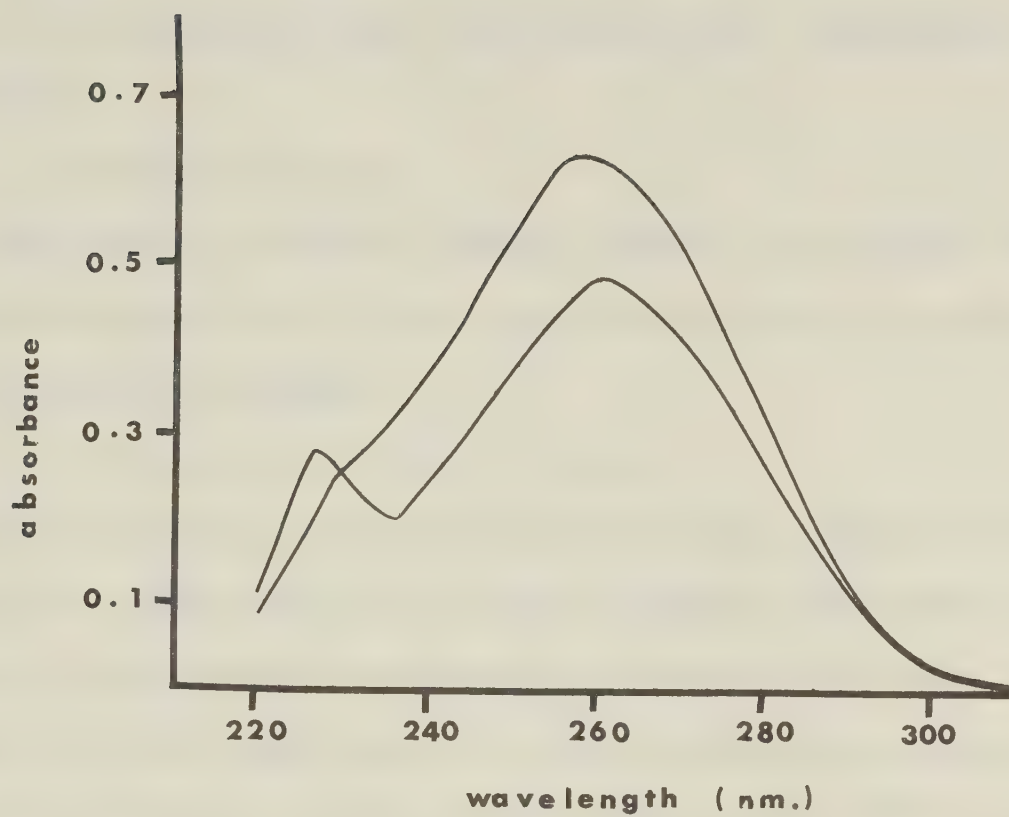




FIGURE 7

Ultraviolet absorption spectrum and melting curve at 260 nm.  
of DNA from V. bubulus (Waterloo) IP 53103.

FIGURE 7







### 1. Biochemical tests.

The biochemical tests employed and their weighted values are shown in Fig. 8. The results of these tests with the OTU's employed are shown in Table 4.

### 2. Computer analysis.

The clustering of the OTU's is shown in Table 5 as resulting from a weighted analysis, and in Table 6 as resulting from an unweighted (or equally weighted) analysis.

### 3. Construction of taxometric maps.

Taxometric maps were constructed from the minimum intercluster discontinuities as determined by the computer analysis, the clusters being linked in the centrality-peripherality sequence as specified. Fig. 9 shows the relative positions of the clusters after the weighted analysis. Three clusters appear, 1) Containing the seven OTU's belonging to the group of facultatively anaerobic corroding bacilli. 2) Containing the four OTU's which are obligate anaerobes and have low base ratios. 3) Containing three of the five Vibrio strains included in the analysis. The remaining 9 OTU's form single member clusters, although clusters 6, 7 and 8, the other species of Bacteroides are placed relatively close to each other, and the remaining two strains of Vibrio are placed close to each other and not very distantly from cluster 3. H. aphrophilus, is the most central cluster, although it is not closely related to any other cluster.

Fig. 10 shows the relative positions of the clusters after the unweighted analysis.





FIGURE 8

Attribute value statistics.

FIGURE 8

ATTRIBUTE VALUE STATISTICS

| ATTRIBUTE                    | MAX    | MIN    | RANGE  | INTRVL CLASSES | LOGMT        |
|------------------------------|--------|--------|--------|----------------|--------------|
| 1 GC% (MEAN OF 2 TO 6 READ   | 652.00 | 280.00 | 373.00 | 10.000         | 38.30 5.2591 |
| 2 GROWTH IN OXYGEN /ANAERO   | 4.00   | 0.0    | 4.00   | 1.000          | 5.00 2.3219  |
| 3 OXIDASE TEST /NEG/POS WI   | 2.00   | 0.0    | 2.00   | 1.000          | 3.00 1.5849  |
| 4 CATALASE /NEG/POS/         | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 5 NITRATE REDUCTION /NEG/P   | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 6 STARCH HYDROLYSIS /NEG/P   | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 7 GELATINE HYDROLYSIS /NEG   | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 8 CASEIN HYDROLYSIS /NEG/P   | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 9 TYROSINE HYDROLYSIS /NEG   | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 10 MALONATE UTILIZATION /NEG | 0.0    | 0.0    | 999.00 | 1.000          | 1000.00 0.0  |
| 11 CITRATE UTILIZATION /NEG  | 0.0    | 0.0    | 999.00 | 1.000          | 1000.00 0.0  |
| 12 PHASE PRODUCTION /NEG/PH  | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 13 RESISTANCE TO SODIUM AZI  | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 14 BILE RESISTANCE /NONE/10  | 2.00   | 0.0    | 2.00   | 1.000          | 3.00 1.5849  |
| 15 INDOLE PRODUCTION /NEG/PH | 0.0    | 0.0    | 999.00 | 1.000          | 1000.00 0.0  |
| 16 HYDROGEN SULFIDE PRODUCT  | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 17 ADENINE DECARBOXYLASE /   | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 18 LYSINE DECARBOXYLASE /NEG | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 19 ORNITHINE DECARBOXYLASE   | 2.00   | 0.0    | 2.00   | 2.000          | 2.00 1.0000  |
| 20 UREASE PRODUCTION /NEG/P  | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 21 AEscULIN HYDROLYSIS /NEG  | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 22 MOTILITY /NEG/POS/        | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |
| 23 ACID PRODUCTION FROM STR  | 1.00   | 0.0    | 1.00   | 1.000          | 2.00 1.0000  |

SIMILARITIES TO BE COMPUTED USING ATTRIBUTES 1 TO 23









TABLE 4 Cont.

## RESULTS OF BIOCHEMICAL TESTS AND BASE RATIOS OF ORGANISMS OF GROUPS I AND II

| OTU                           | <u>B. fragilis</u><br>NCTC 9343 | <u>B. oralis</u> | <u>B. melaninogenicus</u> | <u>V. sputorum</u><br>JV 5 | <u>V. sputorum</u><br>ER 33 | <u>V. bubulus</u><br>10355 | <u>V. foetus</u><br>5850 | <u>V. bubulus</u><br>Waterloo | <u>H. aphrophilus</u><br>NCTC 5886 | <u>Fusobacterium sp.</u> |
|-------------------------------|---------------------------------|------------------|---------------------------|----------------------------|-----------------------------|----------------------------|--------------------------|-------------------------------|------------------------------------|--------------------------|
| Number                        | 14                              | 15               | 16                        | 17                         | 18                          | 19                         | 20                       | 21                            | 22                                 | 23                       |
| %GC                           | 422                             | 430              | 410                       | 469                        | 468                         | 299                        | 320                      | 295                           | 413                                | 653                      |
| Growth in Air                 | -                               | -                | -                         | -                          | -                           | -                          | -                        | -                             | +                                  | -                        |
| Growth in air+CO <sub>2</sub> | -                               | -                | -                         | -                          | -                           | -                          | -                        | -                             | +                                  | -                        |
| Growth in 5%O <sub>2</sub>    | -                               | -                | -                         | -                          | -                           | -                          | -                        | -                             | +                                  | -                        |
| Growth in 1% O <sub>2</sub>   | +                               | +                | -                         | +                          | +                           | +                          | +                        | +                             | +                                  | -                        |
| Growth anaerobic              | +                               | +                | +                         | +                          | +                           | +                          | +                        | +                             | +                                  | +                        |
| Oxidase reaction i.           | -                               | -                | -                         | +                          | +                           | +                          | +                        | +                             | +                                  | -                        |
| Oxidase reaction ii.          | -                               | -                | -                         | +                          | +                           | +                          | +                        | +                             | +                                  | -                        |
| Catalase reaction             | +                               | +                | +                         | +                          | +                           | +                          | +                        | +                             | +                                  | -                        |
| Nitrate reduction             | -                               | -                | -                         | +                          | +                           | +                          | +                        | +                             | +                                  | -                        |
| Starch hydrolysis             | -                               | -                | -                         | -                          | -                           | -                          | -                        | -                             | -                                  | -                        |
| Gelatin hydrolysis            | -                               | -                | -                         | -                          | -                           | -                          | -                        | -                             | -                                  | -                        |
| Casein hydrolysis             | +                               | +                | +                         | -                          | -                           | -                          | -                        | -                             | +                                  | -                        |
| Tyrosine hydrolysis           | -                               | -                | -                         | -                          | -                           | -                          | -                        | -                             | +                                  | -                        |
| Malonate/ $\phi$ -alanine     | -                               | -                | -                         | -                          | -                           | -                          | -                        | -                             | -                                  | -                        |
| Citrate utilization           | -                               | -                | -                         | -                          | -                           | -                          | -                        | -                             | -                                  | -                        |
| DNAse production              | -                               | -                | +                         | -                          | -                           | -                          | -                        | -                             | -                                  | -                        |
| Resistance to azide           | +                               | +                | -                         | -                          | -                           | -                          | -                        | -                             | -                                  | -                        |
| Bile resistance 40%           | -                               | -                | -                         | +                          | +                           | -                          | -                        | -                             | -                                  | -                        |
| Bile resistance 10%           | -                               | -                | -                         | +                          | +                           | +                          | +                        | +                             | -                                  | -                        |
| Indole production             | -                               | -                | -                         | -                          | -                           | -                          | -                        | -                             | -                                  | -                        |
| Arginine decarboxylase        | -                               | +                | +                         | +                          | +                           | +                          | +                        | +                             | +                                  | +                        |
| Lysine decarboxylase          | +                               | -                | -                         | +                          | +                           | -                          | -                        | +                             | -                                  | -                        |
| Ornithine decarboxylase       | -                               | v                | -                         | +                          | +                           | +                          | +                        | +                             | -                                  | -                        |
| Urease production             | -                               | -                | -                         | -                          | -                           | -                          | -                        | -                             | -                                  | -                        |
| Aesculin hydrolysis           | +                               | +                | -                         | -                          | -                           | -                          | -                        | -                             | -                                  | -                        |
| Motility                      | -                               | -                | -                         | +                          | +                           | +                          | +                        | +                             | -                                  | -                        |
| Starch acid.                  | +                               | +                | -                         | +                          | -                           | +                          | -                        | -                             | -                                  | -                        |



TABLE 5

RESULTS FROM COMPUTER ANALYSIS OF BIOCHEMICAL TESTS AND BASE RATIOS, ALL DATA BEING WEIGHTED ACCORDING TO ITS INFORMATION CONTENT

| Cluster No. | OTU No. | Dist.<br>Best<br>Link | OTU<br>Best<br>Link | Name                           |
|-------------|---------|-----------------------|---------------------|--------------------------------|
| 1           | 1       |                       |                     | 333 <u>B. corrodens</u> 10596  |
|             | 2       | 0.0                   |                     | A40 <u>B. corrodens</u> A40/68 |
|             | 5       | 0.00                  | 1                   | 53 P Unknown                   |
|             | 4       | 0.00                  | 5                   | 53 W Unknown                   |
|             | 6       | 0.00                  | 4                   | R27-70                         |
|             | 7       | 0.0                   | 6                   | S-209                          |
|             | 3       | 0.00                  | 6                   | B-916                          |
|             | 18      | 0.26                  | 3                   | ER 33 <u>V. sputorum</u>       |
| 2           | 10      |                       |                     | 4053 Unknown UAH-2             |
|             | 11      | 0.00                  |                     | B-912                          |
|             | 8       | 0.03                  | 10                  | EDMH-1 Misericordia            |
|             | 9       | 0.04                  | 11                  | 4282 Lane UAH-1                |
|             | 21      | 0.34                  | 8                   | Waterloo <u>V. bubulus</u>     |





TABLE 5 cont'd

| Cluster No. | OTU No. | Dist.<br>Best<br>Link | OTU<br>Best<br>Link | Name                       |
|-------------|---------|-----------------------|---------------------|----------------------------|
| 3           | 19      |                       |                     | 10355 <u>V. bubulus</u>    |
|             | 20      | 0.05                  |                     | 5850 <u>V. foetus</u>      |
|             | 21      | 0.08                  | 19                  | Waterloo <u>V. bubulus</u> |
|             | 18      | 0.12                  | 21                  | ER 33 <u>V. sputorum</u>   |

## Isolated OTU's (single member clusters).

|    |    |  |  |                                 |
|----|----|--|--|---------------------------------|
| 4  | 12 |  |  | 3936 Unknown                    |
| 5  | 13 |  |  | 4482 Unknown                    |
| 6  | 14 |  |  | NCTC 9343 <u>B. fragilis</u>    |
| 7  | 15 |  |  | <u>B. oralis</u>                |
| 8  | 16 |  |  | <u>B. melaninogenicus</u>       |
| 9  | 17 |  |  | JV 5 <u>V. sputorum</u>         |
| 10 | 18 |  |  | ER 33 <u>V. sputorum</u>        |
| 11 | 22 |  |  | NCTC 5886 <u>H. aphrophilus</u> |
| 12 | 23 |  |  | <u>Fusobacterium</u> sp.        |



TABLE 6

RESULTS OF COMPUTER ANALYSIS OF BIOCHEMICAL TESTS AND BASE RATIOS  
ALL DATA BEING EQUALLY WEIGHTED

| Cluster No. | OTU No. | Dist.<br>Best<br>Link | OTU<br>Best<br>Link | Name                            |
|-------------|---------|-----------------------|---------------------|---------------------------------|
| 1           | 1       |                       |                     | <u>B. corrodens</u> 10596       |
|             | 2       | 0.0                   |                     | <u>B. corrodens</u> A40/68      |
|             | 5       | 0.00                  | 1                   | 53 P Unknown                    |
|             | 4       | 0.00                  | 5                   | 53 W Unknown                    |
|             | 6       | 0.00                  | 4                   | R 27/70                         |
|             | 7       | 0.00                  | 6                   | S 209                           |
|             | 3       | 0.00                  | 6                   | B-916                           |
|             | 21      | 0.22                  | 3                   | Waterloo <u>V. bubulus</u>      |
| 2           | 10      |                       |                     | 4053 Unknown UAH-2              |
|             | 11      | 0.00                  |                     | B-912                           |
|             | 8       | 0.02                  | 10                  | EDMH-1 Misericordia             |
|             | 9       | 0.05                  | 11                  | 4282 Lane UAH-1                 |
|             | 22      | 0.35                  | 9                   | <u>H. aphrophilus</u> NCTC 5886 |



TABLE 6 cont'd

| Cluster No. | OTU No. | Dist.<br>Best<br>Link | OTU<br>Best<br>Link | Name                       |
|-------------|---------|-----------------------|---------------------|----------------------------|
| 3           | 18      |                       |                     | ER 33 <u>V. sputorum</u>   |
|             | 21      | 0.05                  |                     | Waterloo <u>V. bubulus</u> |
|             | 17      | 0.10                  | 21                  | JV 5 <u>V. sputorum</u>    |
|             | 19      | 0.10                  | 17                  | 10355 <u>V. bubulus</u>    |
|             | 20      | 0.05                  | 19                  | 5850 <u>V. foetus</u>      |
|             | 3       | 0.22                  | 21                  | B-916                      |

## Isolated OTU's (single member clusters).

|    |    |  |  |                              |
|----|----|--|--|------------------------------|
| 4  | 12 |  |  | 3036 Unknown                 |
| 5  | 13 |  |  | 4482 Unknown                 |
| 6  | 14 |  |  | <u>B. fragilis</u> NCTC 9343 |
| 7  | 15 |  |  | <u>B. oralis</u>             |
| 8  | 16 |  |  | <u>B. melaninogenicus</u>    |
| 9  | 22 |  |  | <u>H. aphrophilus</u>        |
| 10 | 23 |  |  | <u>Fusobacterium</u> sp.     |







FIGURE 9

Taxometric map of clusters resulting from weighted computer analysis of biochemical data and base ratios of organisms of Groups I and II.

| Cluster membership index:             | Cluster |
|---------------------------------------|---------|
| 1. <u>B. corrodens</u> NCTC 10596 333 | 1       |
| 2. <u>B. corrodens</u> A40/68         | 1       |
| 3. B-916                              | 1       |
| 4. 53 W                               | 1       |
| 5. 53 P                               | 1       |
| 6. R 27/70                            | 1       |
| 7. S 209                              | 1       |
| 8. EDMH-1 Misericordia                | 2       |
| 9. UAH-1 Lane 4282                    | 2       |
| 10. UAH-2 4053                        | 2       |
| 11. B-912                             | 2       |
| 12. 3936                              | 4       |
| 13. 4482                              | 5       |
| 14. <u>B. fragilis</u> NCTC 9343      | 6       |
| 15. <u>B. oralis</u>                  | 7       |
| 16. <u>B. melaninogenicus</u>         | 8       |
| 17. JV. 5 <u>V. sputorum</u>          | 9       |
| 18. ER 33 <u>V. sputorum</u>          | 10      |
| 19. 10355 <u>V. bubulus</u>           | 3       |
| 20. 5850 <u>V. foetus</u>             | 3       |
| 21. Waterloo <u>V. bubulus</u>        | 3       |
| 22. <u>H. aphrophilus</u>             | 11      |
| 23. <u>Fusobacterium</u> sp.          | 12      |

FIGURE 9

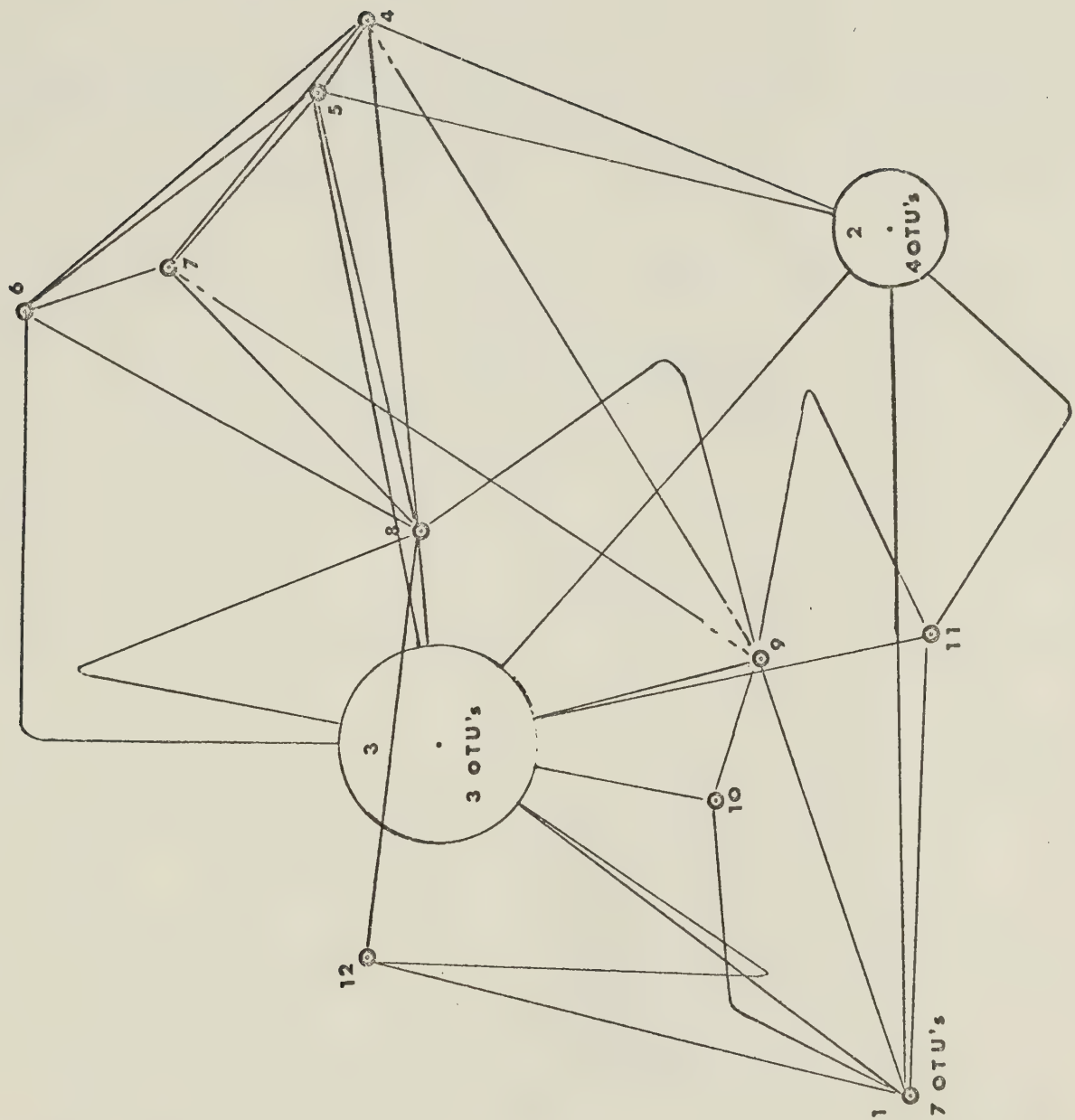






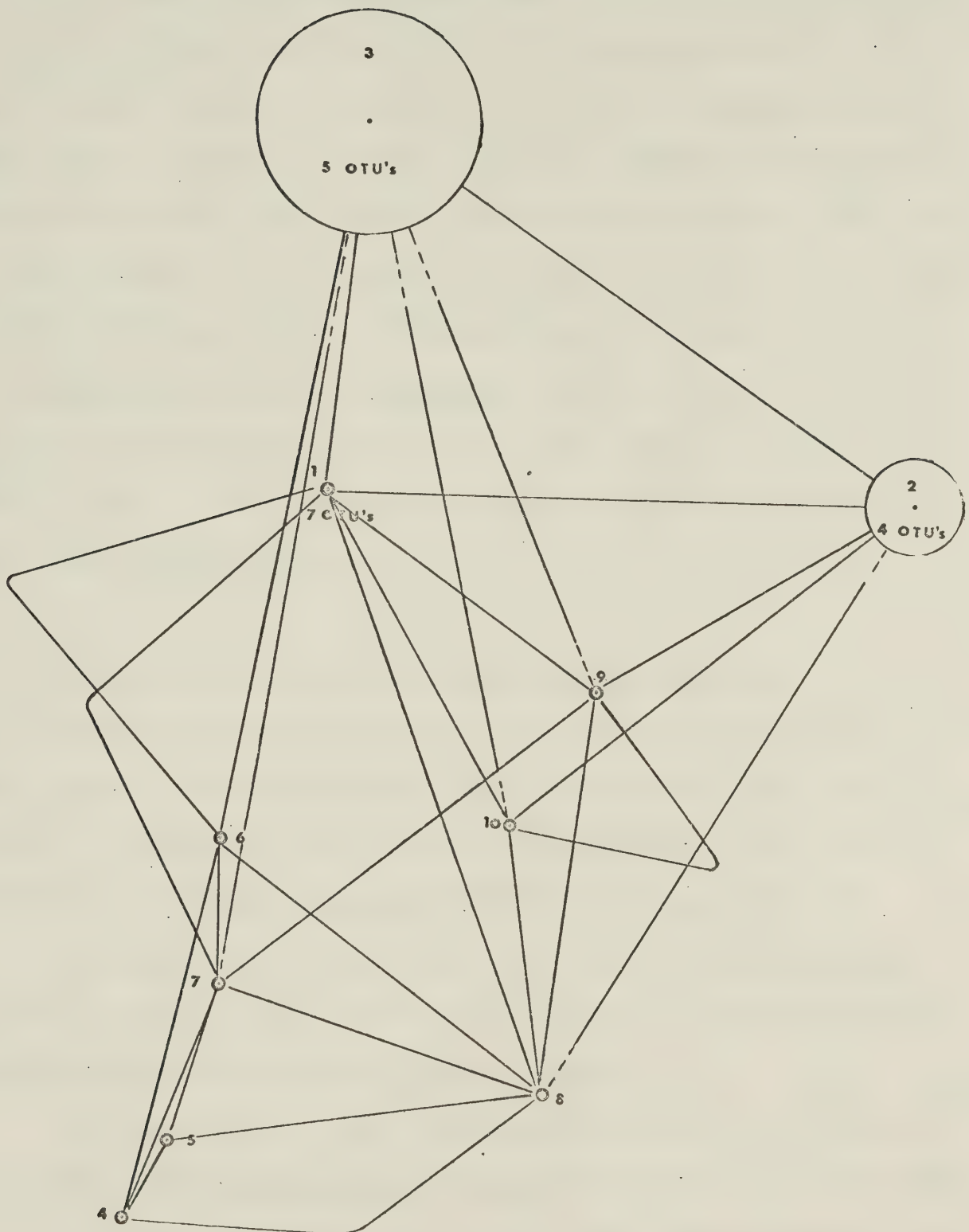
FIGURE 10

Taxometric map of clusters resulting from unweighted computer analysis of biochemical data and base ratios of organisms of Groups I and II.

| Cluster membership index:                    | Cluster |
|----------------------------------------------|---------|
| 1. <u>B. <i>corrodens</i></u> NCTC 10596 333 | 1       |
| 2. <u>B. <i>corrodens</i></u> A/40 /68       | 1       |
| 3. B-916                                     | 1       |
| 4. 53 W                                      | 1       |
| 5. 53 P                                      | 1       |
| 6. R 27/70                                   | 1       |
| 7. S-209                                     | 1       |
| 8. EDMH-1 Misericordia                       | 2       |
| 9. UAH-1 Lane 4482                           | 2       |
| 10. UAH-2 4053                               | 2       |
| 11. B-912                                    | 2       |
| 12. 3936                                     | 4       |
| 13. 4482                                     | 5       |
| 14. <u>B. <i>fragilis</i></u> NCTC 9343      | 6       |
| 15. <u>B. <i>oralis</i></u>                  | 7       |
| 16. <u>B. <i>melaninogenicus</i></u>         | 8       |
| 17. JV 5 <u>V. <i>sputorum</i></u>           | 3       |
| 18. ER 33 <u>V. <i>sputorum</i></u>          | 3       |
| 19. 10355 <u>V. <i>bubulus</i></u>           | 3       |
| 20. 5850 <u>V. <i>foetus</i></u>             | 3       |
| 21. Waterloo <u>V. <i>bubulus</i></u>        | 3       |
| 22. <u>H. <i>aphrophilus</i></u>             | 9       |
| 23. <u>Fusobacterium</u> sp.                 | 10      |



FIGURE 10





# V. Determination of base ratios for organisms belonging to the Micrococcus-Staphylococcus complex.

The base ratios obtained for members of this complex are shown in Table 7. Those organisms classified as Micrococcus species which showed higher degrees of similarity with species of Staphylococcus were found also to have low, more typically staphylococcal base ratios in most instances. The organisms used which had previously been subjected to base ratio determinations by Rosypal, Rosypalová and Hořejš<sup>v</sup> (1966) were found to have intermediate base ratios typical of neither the Staphylococcus nor the Micrococcus species. A representative absorption spectrum and melting curve for DNA from M. halophilus is shown in Fig. 11, and an absorption spectrum and melting curve of a DNA from M. cyaneus CCM 856 is shown in Fig. 12.

# VI. Determination of base ratios for Pasteurella species.

Values obtained for the base ratios of the Pasteurella strains and species listed as Group IV in the methods section, are shown in Table 8. A representative absorption spectrum and melting curve of DNA extracted from P. multocida MU3004-58 are shown in Fig. 13.

All the values obtained for base contents of the Pasteurella strains are very similar, with the exception of P. pseudotuberculosis and the organism tentatively called P. pfaffii.

The base ratios of the enterobacteria included for comparison with those of the Pasteurella species, are very similar to those values reported in the literature, the Proteus species characteristically having a lower GC content than the other members of the Enterobacteriaceae.



TABLE 7

BASE RATIO DETERMINATIONS FOR MEMBERS OF THE STAPHYLOCOCCUS-MICROCOCCUS COMPLEX, (GROUP IV).

| Organisms                              | Mean GC% | Range<br>% GC | Mean T <sub>m</sub> | in Buffer | Lit.<br>value     | Ref.        |
|----------------------------------------|----------|---------------|---------------------|-----------|-------------------|-------------|
| <u>M. tetragenus</u>                   | 31.1     | 0.6           | 82.00               | SSC       |                   |             |
| <u>M. halophilus</u>                   | 31.1     | 0.2           | 82.00               | SSC       |                   |             |
| <u>M. cyaneus</u><br>NCIB 9148         | 33.5     | 1.0           | 83.00               | SSC       | 63%               | (1)         |
| <u>M. cyaneus</u> *<br>CCM 856         | 66.5     | 1.0           | 96.50               | SSC       | 63%               | (1)         |
| <u>M. freudenreichii</u>               | 38.0     | 2.0           | 85.25               | SSC       | 59%<br>(ATCC 507) | (1)         |
| <u>M. aurantiacus</u>                  | 29.4     | 1.2           | 81.50               | SSC       |                   |             |
| <u>M. candidus</u>                     | 29.2     | 3.0           | 81.50               | SSC       | 30%               | (1)         |
| <u>M. roseus</u><br>NCIB 8175          | 70.2     | 1.2           | 88.75               | SSC/5     | 68%-75%           | (8)         |
| <u>M. roseus</u><br>CCM 706            | 72.0     | 2.0           | 89.50               | SSC/5     | 72.3%             | (95)        |
| <u>M. varians</u><br>CCM 884           | 70.9     | 0.5           | 85.00               | SSC/10    | 69.3%             | (95)        |
| <u>M. conglomeratus</u><br>NCIB 2677   | 63.6     | 1.3           | 85.50               | SSC/5     | 63.00%            | (1)         |
| <u>M. luteus</u> *<br>CCM 852          | 70.8     | 0.6           | 89.00               | SSC/5     | 64%<br>71%        | (1)<br>(95) |
| <u>M. violagabriellae</u><br>NCTC 9865 | 31.3     | 2.1           | 82.00               | SSC       | 30.0              | (1)         |





TABLE 7 cont'd

| Organisms                      | Mean GC% | Range<br>% GC | Mean T <sub>m</sub> | in Buffer | Lit.<br>value | Ref. |
|--------------------------------|----------|---------------|---------------------|-----------|---------------|------|
| <u>M. aquivivus</u><br>CCM 316 | 50.2     | 1.0           | 89.75               | SSC       | 47%-51%       | (8)  |
| <u>M. varians</u><br>CCM 250   | 52.9     | 1.6           | 90.75               | SSC       | 54.2%         | (95) |

\* These organisms were also subjected to determination of %GC using SSC diluted 1/2, 1/5 and 1/10.





FIGURE 11

Ultraviolet absorption spectrum and melting curve at 260 nm.  
of DNA from M. halophilus.

FIGURE 11

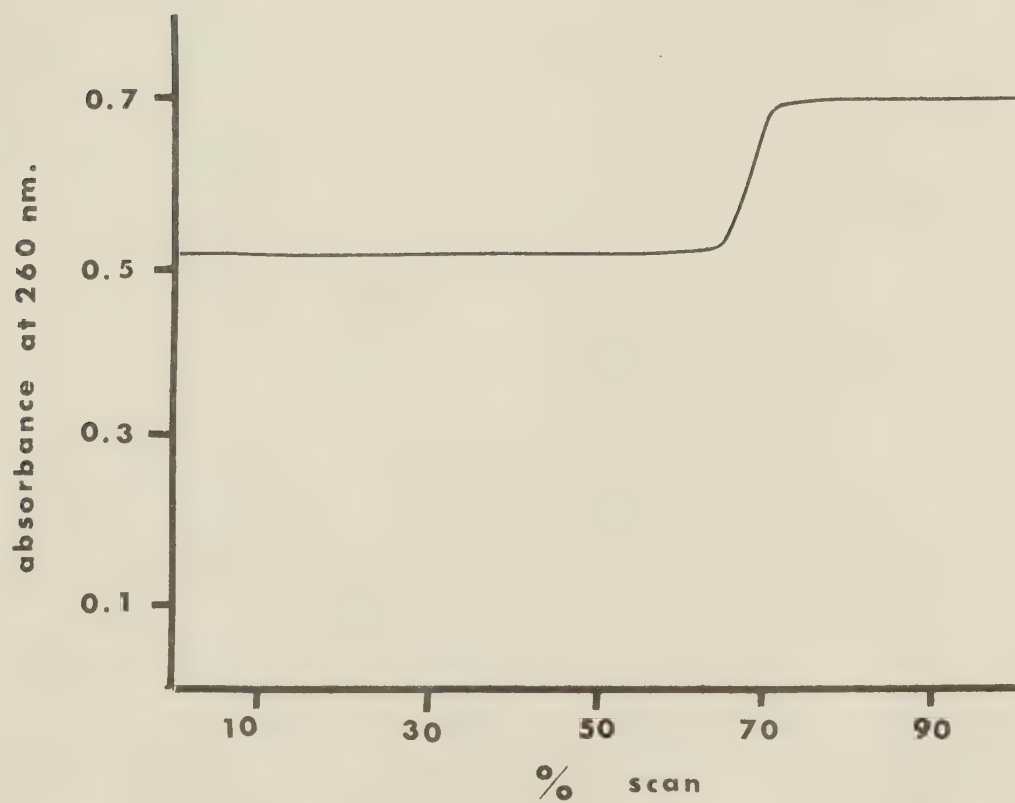
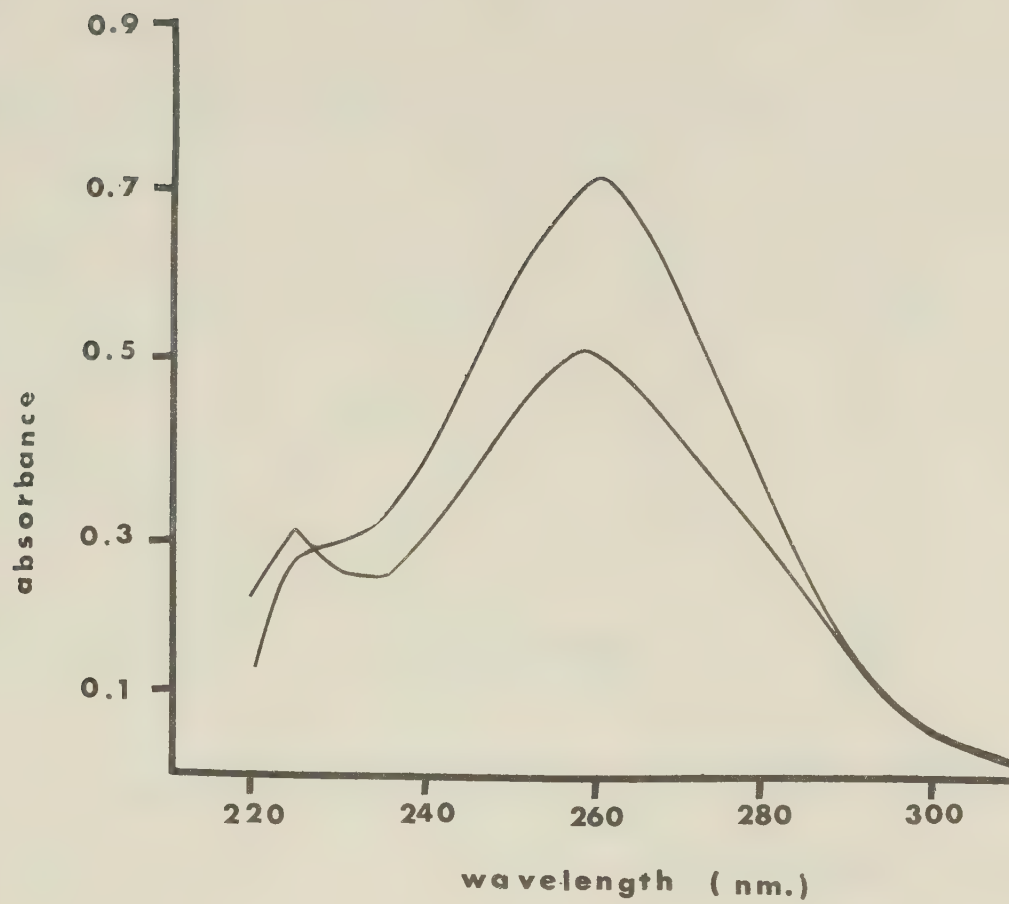








FIGURE 12

Ultraviolet absorption spectrum and melting curve at 260 nm.  
of DNA from M. cyaneus CCM 856.

FIGURE 12

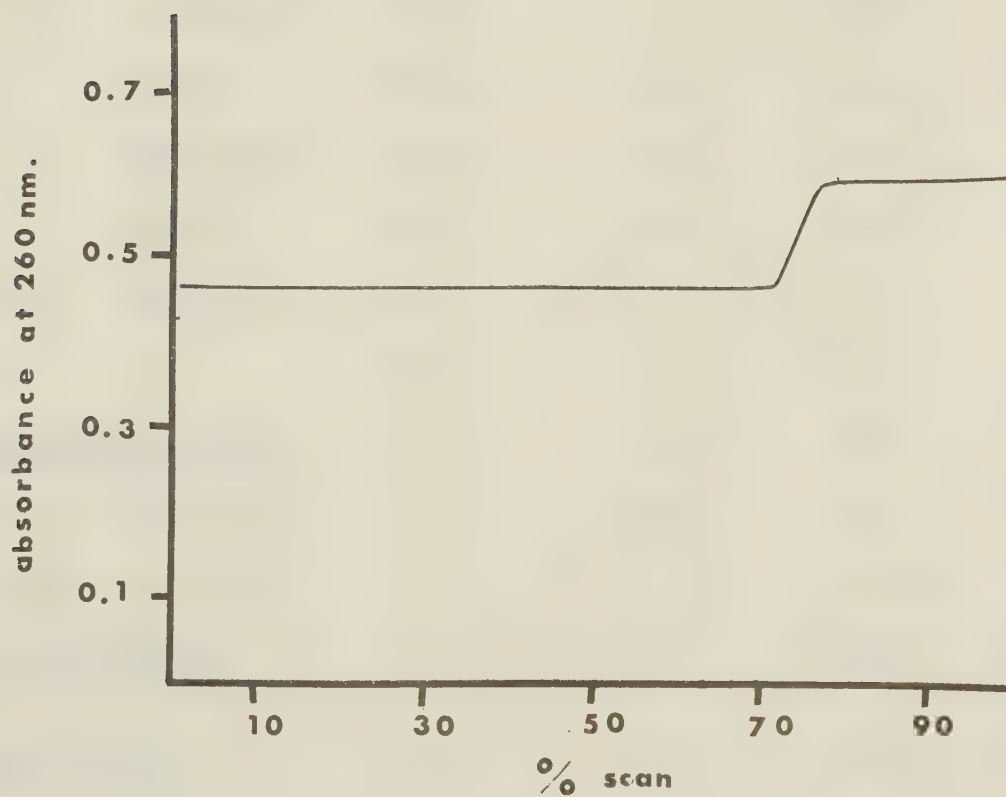
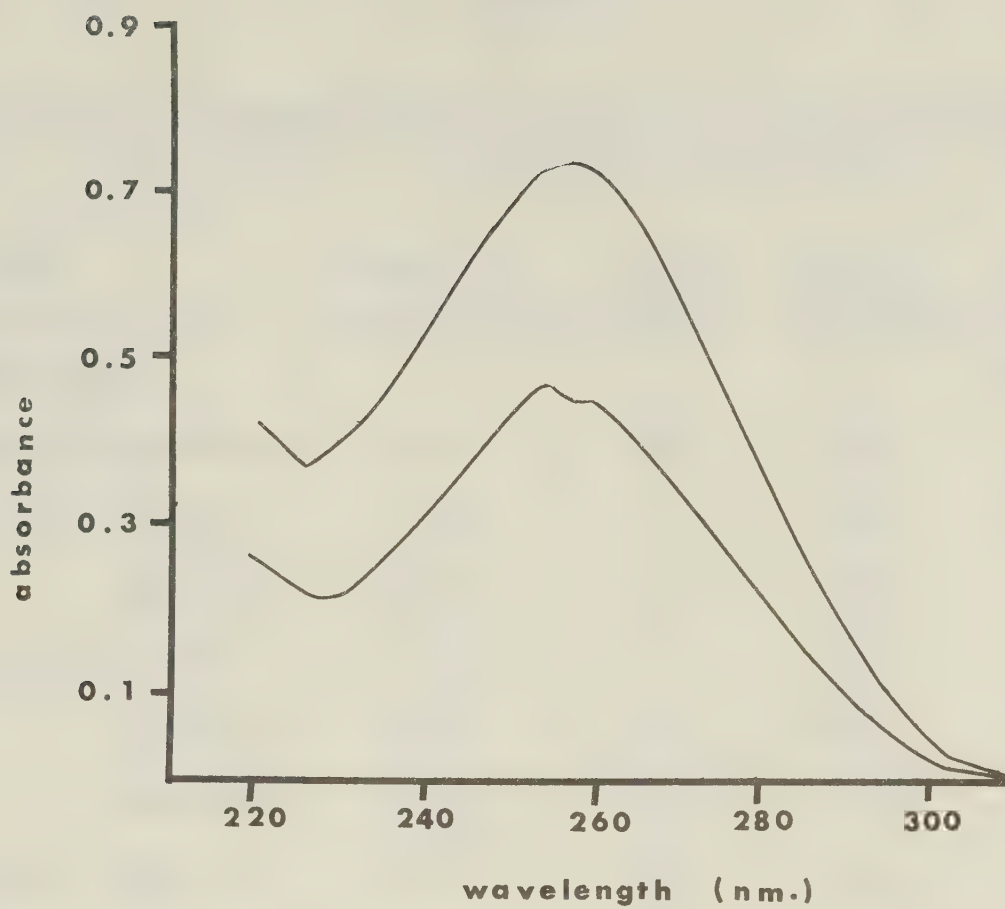




TABLE 8

BASE RATIOS OF PASTEURELLA STRAINS (GROUP III) DETERMINED IN SSC

| Organism                         | Mean GC % | Range % GC | Mean T <sub>m</sub> in SSC. | Lit. value | Ref. |
|----------------------------------|-----------|------------|-----------------------------|------------|------|
| <u>P. multocida.</u>             |           |            |                             | 36-37%     | (4)  |
| Group I(A) 10456-56              | 41.7      | 0.6        | 86.40                       |            |      |
| 5568-61                          | 41.4      | 1.0        | 86.25                       |            |      |
| M4631-60                         | 40.8      | 0.5        | 86.00                       |            |      |
| Group II(D) 675-59               | 39.0      | 1.2        | 85.25                       |            |      |
| 10955-55                         | 40.8      | 0.5        | 86.00                       |            |      |
| 11085-55                         | 40.9      | 1.0        | 86.00                       |            |      |
| Group III 5228-58                | 41.1      | 0.0        | 86.10                       |            |      |
| MU3004-58                        | 40.9      | 1.2        | 86.00                       |            |      |
| M6395                            | 42.0      | 0.8        | 86.50                       |            |      |
| MU1109-61                        | 40.2      | 0.5        | 85.75                       |            |      |
| M2373                            | 41.5      | 0.0        | 86.25                       |            |      |
| Group IV M191-60                 | 40.5      | 1.8        | 85.75                       |            |      |
| Type C*                          | 40.2      | 1.5        | 85.75                       |            |      |
| <u>P. pseudotuberculosis</u>     | 48.2      | 1.0        | 89.00                       |            |      |
| <u>P. ureae</u> MU7572-61        | 40.9      | 0.0        | 86.00                       |            |      |
| <u>P. ureae</u> MU5105-60        | 40.9      | 0.2        | 86.00                       |            |      |
| <u>P. pneumotropica</u> M3913-66 | 40.9      | -          | 86.00                       | 42%        | (12) |
| <u>P. haemolytica</u> M1583-62   | 39.6      | 0.4        | 86.00                       | 40-42%     | (85) |





TABLE 8. cont'd

| Organism                          | Mean GC % | Range<br>% GC | Mean T <sub>m</sub><br>in SSC | Lit.<br>value | Ref. |
|-----------------------------------|-----------|---------------|-------------------------------|---------------|------|
| ? <u>P. pfaffii</u> SC 1-64       | 57.3      | 0.6           | 92.75                         |               |      |
| MU 14249-64                       | 51.2      | 0.8           | 90.25                         |               |      |
| MU 7120-69                        | 75.5      | 1.2           | 88.00                         |               |      |
| <u>Proteus vulgaris</u>           | 42.1      | 2.0           | 86.50                         | 36-41%        | (58) |
| <u>Klebsiella</u> sp.<br>U 113-70 | 57.5      | 1.8           | 92.50                         | 52-56%        | (79) |
| <u>Enterobacter</u> sp. #62       | 54.2      | 0.4           | 91.50                         |               |      |
| <u>E. coli</u> B.                 | 51.8      | -             | 90.50                         |               |      |

\*T<sub>m</sub> determined in SSC, SSC/2, SSC/5 and SSC/10

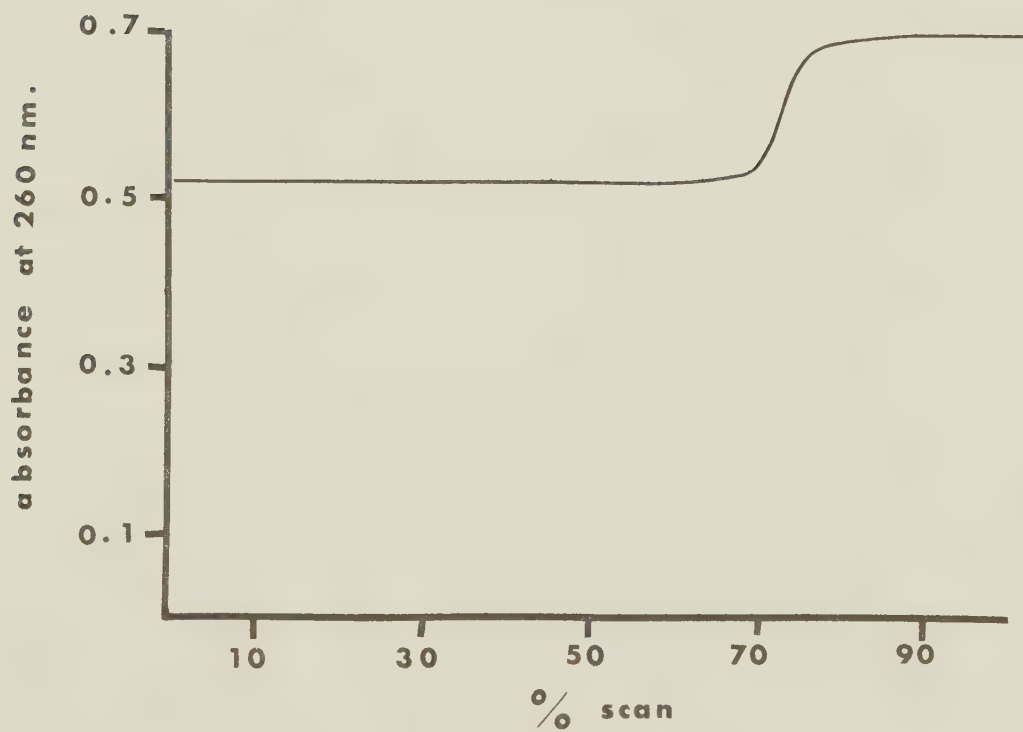
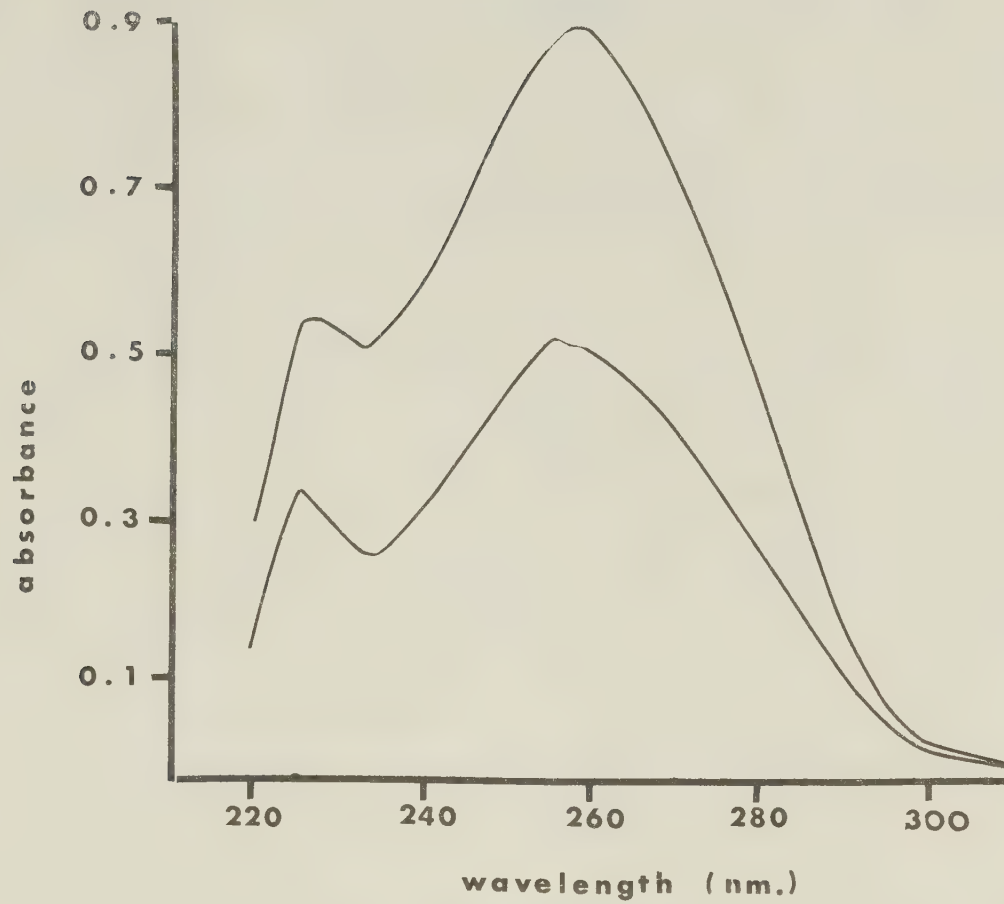




FIGURE 13

Ultraviolet absorption spectrum and melting curve at 260 nm.  
of DNA from P. multocida MU 3004-58.

FIGURE 13





CONCLUSIONS





## CONCLUSION

I. Base ratios and relationships of organisms tentatively designated B. corrodens (Group I), were investigated and showed base ratios which fell into three distinct groups:

- i) the facultatively anaerobic strains, which have base ratios of 56.9%-57.9% GC: B. corrodens NCTC 10596 333/ 54-55, B. corrodens A40/68, B-916, 53W, 53P, R 27/70 and S-209;
- ii) the obligately anaerobic strains which have base ratios of 28.0%-29.7% GC, strains UAH-1 (Lane), UAH-2 (4053), EDMH-1 (Misericordia), and B-912;
- iii) the obligately anaerobic strains which have 38.3%-39.3% GC, strains 3936 and 4482.

Within each group the base ratios are consistent and there would seem to be no reason to regard the obligately anaerobic strains as biotypes of the facultative strains.

II. Because of possible confusion on biochemical grounds between these corroding bacilli and other Gram-negative anaerobic or facultatively anaerobic organisms (Group II), base ratios of members of this latter group were investigated. H. aphrophilus, once suggested as being related to the organism called B. corrodens was found to have a GC content of 41%, which is in keeping with the other species of Haemophilus, but different from the base content of the facultatively anaerobic corroding bacilli. Other members of the genus Bacteroides also had base ratios of 40%-43% GC in keeping with the previously reported value for B. insolitus (4) but not with the value of 31% GC found for B. clostrid-



iformis. The members of the genus Bacteroides are anaerobic, and the base ratios found for B. fragilis, B. oralis and B. melaninogenicus are similar to those found for two anaerobic strains of corroding bacilli, 3936 and 4482 (38.3%-38.9% GC). In certain keys V. sputorum is distinguished from B. corrodens only in its being motile, however its base ratio of 46.8% GC is correspondant with none of the groups of corroding bacilli, and is in good agreement with the base ratios of those organisms included in the genus Vibrio by Sebald and Veron (1963), which have c.47% GC. Anaerobic, non-fermentative species of Vibrio, proposed by the same authors as members of the genus Campylobacter, were found to have values for GC contents of 29.5% for V. bubulus, and 32% for V. foetus.

III. The patterns of phenetic relationships which exist between micro-organisms can be described by summarizing the mutual similarities of organisms by cluster analysis. Computation of relative proximities between items, and graphic display of inter-cluster discontinuity, and intra-cluster variation was achieved for the Gram-negative, anaerobic or facultatively anaerobic organisms described as Groups I and II, using biochemical data and base ratios in the TAXMAP programme.

When data were analysed after being weighted according to information content, three distinct clusters occurred. Cluster I contained all seven strains of facultatively anaerobic corroding bacilli, with the maximum relative distance between the two most distant OTU's being only 0.002, indicating that the strains were almost identical. Cluster 2 contained the four obligately anaerobic strains of corroding bacilli which had base ratios of 28.0%-29.7% GC. Cluster 3 included three strains of Vibrio species (Campylobacter species) V. foetus and



V. bubulus with base ratios of 29.5%-32% GC. Quite close to this cluster were two strains of V. sputorum with base ratios of 46.8% GC, but these and the remaining OTU's formed single member clusters. Of the three non-corroding species of Bacteroides investigated, B. fragilis and B. oralis were quite closely related, and both were relatively more distantly related to B. melaninogenicus. Strains of anaerobic corroding bacilli 3936 and 4482 were also quite closely related to these other species of Bacteroides, and also had similar base ratios. The relative distances between these organisms could be shown undistortedly in two dimensions on the map. H. aphrophilus, although the most centrally located OTU was not closely related to any other OTU, nor was the strain of Fusobacterium.

When the same data were analysed equally weighted, again three clusters appeared with similar relative inter-cluster distances to those obtained with the weighted analysis, although the centrality-peripherality perspective was somewhat altered. Clusters 1 and 2 contained the same OTU's as before and Cluster 3 was now found to encompass all five strains of Vibrio, including the strains of V. sputorum with the higher base ratios. This correlation reflects the original broad scope of the genus Vibrio as classified on purely biochemical and morphological grounds. The position of the Bacteroides species and strains 3936 and 4482 were conserved in approximately the same relation as before with the weighted data, and the comparative unrelatedness of both H. aphrophilus and the strain of Fusobacterium were again shown.

IV. The previous investigations of the Staphylococcus-Micrococcus complex have shown two distinct groups in these organisms. Work in this laboratory showed a correlation between the generic status of members of







this complex, and their cytochrome content: members of the genus Micrococcus having usually cytochromes a, b, and c, and members of the genus Staphylococcus having only cytochromes a and b. Exceptions to these patterns were strains labelled as Micrococcus species which usually also showed aberrant biochemical patterns and were placed in Staphylococcus rather than Micrococcus clusters on computer analysis. When these organisms named as Micrococcus species but showing Staphylococcus-like cytochrome patterns were investigated with regard to their base ratios, all but one showed low, Staphylococcus-like base ratios of 29%-39% GC, suggesting again that they were of the genus Staphylococcus rather than Micrococcus. The exception to this was found in M. cyaneus CCM 856 which was reported to lack cytochrome c, but was found to have a base ratio of 66.5% GC. However this organism is heavily pigmented and visual identification of its cytochromes is difficult, and another strain of M. cyaneus, NCIB 9148 which also lacked cytochrome c was found to have a base ratio of 33.5% GC. Thus it would appear that the two strains are not representative of the same species, the former resembling members of the genus Micrococcus, and the latter members of the genus Staphylococcus.

V. The determination of base ratios for members of the genus Pasteurella revealed great consistency in base content between strains of P. multocida and these strains which could be differentiated on serological grounds were indistinguishable in terms of their GC content. No correlation could be found in the very narrow range (c. 2%) of base ratios found, between serological type and % GC. The base ratios found for the strains of P. multocida were higher than those previously



reported (4), though the earlier determinations had used techniques of chromatography. The value found for P. haemolytica (39.6% GC) corresponds well with that reported by Mraz (1969) of 40% GC, and values of 40.9% GC for strains of P. ureae reflect the biochemical similarity of this organism and P. haemolytica.

Of the Pasteurella species investigated, only P. pseudotuberculosis had a significantly higher base ratio (48.2% GC) which is in keeping with its proposed inclusion in the genus Yersinia (van Loghem, 1946) with P. pestis which has been found to have a base ratio of 46%-47% GC (4).

The organism tentatively designated P. pfaffii was found to have a base ratio of 57.3% GC, which is unlike any value reported for any Pasteurella species, and the organism is unlikely to be a member of the genus. No value for the base ratio of P. pfaffii has been reported.

The values found for the base ratios of the enterobacteria included for purposes of comparison were very similar to those previously reported.



## VII

## DISCUSSION



## DISCUSSION

Classification of microorganisms on the basis of predominantly negative results of biochemical tests can give little indication of the metabolic characteristics and capabilities of those organisms. Useful information about the similarity and likelihood of relationship between such organisms can be obtained by investigation of their base ratios.

The taxonomic status of the Gram-negative corroding bacilli here investigated is one in which organisms being relatively inert in the conventional diagnostic biochemical tests have been grouped together on the basis of an obvious, but not necessarily specific criterion, their pitting of agar. However, base ratios of these organisms show distinct groupings, which may also be correlated with certain biochemical properties. Facultatively anaerobic strains, which resemble Henrikson's type strain, produce arginine, lysine and ornithine decarboxylases, do not hydrolyse gelatin, do not produce urease and have base ratios of 56.9%-57.9% GC. Obligately anaerobic strains do not produce arginine, lysine or ornithine decarboxylases, do hydrolyse gelatin, and do produce urease; these strains have base ratios of 28.0%-29.7% GC.

The most valuable application of GC ratios as a taxonomic criterion seems to be in its use in conjunction with an Adansonian type of analysis based on overall similarity. In various Gram-negative obligate and facultative anaerobes here investigated, computer analysis of biochemical data located OTU's into clusters, the members of which also had very similar base ratios. Two distinct groups of corroding bacilli occurred, apparently unrelated to each other and to other species of





Bacteroides and Vibrio. Two other agar-pitting strains, obligate anaerobes 3936 and 4482 showed close affinity for neither of the clusters of other corroding bacilli, but were located by the computer analysis in fairly close proximity to other species of Bacteroides and these strains also had base ratios (38-39% GC) which were close to those of other Bacteroides species (c. 40-42% GC). The corroding bacilli with base ratios of 56.9%-57.9% GC and of 28.0%-29.7% do not fit into any of the genera at present defined. The identification of either group as a species of the genus Bacteroides is dubious, considering the disparity of base ratios between these groups and most other Bacteroides species, which have c. 40% GC. Also the inclusion of the two groups as species within one new genus is obviously unsatisfactory.

Other genera which have base ratios similar to those of the facultatively anaerobic corroding bacilli are Acetobacter, Aeromonas, Azotobacter, Bifidobacterium, Brucella, certain strains of Pseudomonas, and various species of Enterobacteriaceae, particularly species of Aerobacter and Serratia. These genera are different in morphological and biochemical characteristics from the corroding bacilli. In view of the lack of a genus to accommodate these strains it would seem reasonable to propose a new genus defined as Gram-negative rods, non-motile, facultatively anaerobic, oxidase positive, catalase negative, producing nitrite from nitrate, not fermenting glucose, indole negative, with base ratios of 57%-58% GC. Colonies of these organisms frequently produce small pits in the agar. The type species would be NCTC 10596 (333 /54-55), which was one of Eiken's original strains.



The only organisms known to have base ratios similar to those of the group of anaerobic agar-pitting organisms B-912, EDMH-1 (Misericordia), UAH-1 (Lane) and UAH-2 (4053) are species of the genera Staphylococcus, Clostridium and Mycoplasma, and Ramibacterium ramosum, all of which are obviously different in their morphological and biochemical characteristics from the Gram-negative corroding bacilli. Certain species of Vibrio (Campylobacter) have similar base ratios, but are serologically and biochemically distinct. Certain strains of Spirillum linum, Fusobacterium fusiforme, and Bacteroides clostridiformis have been found to have similar base ratios but more information is required about their other characteristics. However, the properties of this group are less fully described than those of the facultatively anaerobic strains, and certain characteristics, such as the formation of spreading colonies on agar plates and resemblances to certain Myxobacter species which are not usually found in organisms present in the human flora, need further investigation. It may be necessary to include these organisms in another genus of Gram-negative rods which are non-flagellated, obligately anaerobic, oxidase positive, catalase negative, produce nitrites from nitrites, do not ferment glucose, are indole negative and have base ratios of 28%-30% GC. The colonies of such organisms may show peripheral agar pitting, and sometimes give rise to a thin spreading growth on the surface of the agar. In many features they resemble certain of the strains described by Khairat (1967) as Bacteroides corrodens. They also resemble some strains included in the group F-3 fastidious anaerobes, described in the CDC Anaerobic Bacteriology Manual (Dowell and Hawkins, 1968).



The application of base ratios to the classification of the Staphylococcus-Micrococcus complex has been made by many workers, and its usefulness in confirming the position of certain organisms whose generic status on computer analysis of biochemical data differs from their previous classification, has been well established (1, 9, 87). Results obtained in this investigation also corroborated the analysis of biochemical data which placed certain organisms hitherto regarded as Micrococcus species in the genus Staphylococcus.

However, the usefulness of base ratios in differentiation of strains and species of an homogenous genus is questionable. As with many members of the Enterobacteriaceae, the strains of Pasteurella multocida and other Pasteurella species here investigated were indistinguishable by their base content, while serological methods could be used to detect several groups. However, differences in base ratios do reaffirm the proposed division of the genus Pasteurella into the genera Pasteurella, Yersinia and Francisella. The main diagnostic use of base ratios in this instance would seem to be the recognition of Pasteurella-like organisms as non-Pasteurella species, as in the case of the organism tentatively called P. pfaffii, which had a base ratio far higher than any known Pasteurella species, as did the unidentified Gram negative rod MU 14249-64 which showed Pasteurella-like biochemical characteristics, thus these organisms are unlikely to be Pasteurella species.







VIII

REFERENCES



## REFERENCES

1. AULETTA, A.E. and KENNEDY, E.R. (1966). Deoxyribonucleic acid content in some members of the Micrococcaceae. J. Bact. 92, 28.
2. BAILLIE, W.E. (1969). Ph.D. Thesis, Kansas State University.
3. BASDEN, E.H. II, TOURTELLOTTÉ, M.E., PLASTRIDGE, W.N. and TUCKER, J.S. (1968). Genetic relations among bacteria classified as Vibrios. J. Bact. 95, 439.
4. BELOZERSKY, A.N. and SPIRIN, A.S. (1960). The Nucleic Acids, ed. by E. Chargaff and J.N. Davidson, vol. 3, p. 147. New York, Academic Press.
5. Bergey's Manual of Determinative Bacteriology (1957). 7th ed. Ed. R.S. Breed, E.G.D. Murray and N.R. Smith. London. Balliere, Tindall and Cox.
6. BIBERSTEIN, E.L. and FRANCIS, C.K. (1968). Nucleic homologies between A and T types of Pasteurella haemolytica. J. Med. Microbiol. 1, 105.
7. BLAZEVIK, D.J. (1968). Improved Indole-Motility medium. Appl. Microbiol. 16, 668.
8. BOHÁČEK, J., KOCUR, M. and MARTINEC, T. (1967). DNA base composition and taxonomy of the genus Micrococcus. J. gen. Microbiol. 46, 369.
9. BOHÁČEK, J. and MRAZ, O. (1967). Basengehalt der Desoxyribonukleinsäure bei den Arten P. haemolytica, A. ligniersi und A. equuli. Zentbl. f. Bakt. I Abt. Orig. 202, 468.
10. BOUISSET, L., BREUILLARD, J. and MICHEL, G. (1963). Étude de l'ADN chez les Actinomycetales. Ann. Inst. Past. 104, 488.
11. BOUISSET, L., BREUILLARD, J. and MICHEL, G. (1963). Étude de l'ADN chez les Actinomycetales. Comparaison entre les valeurs du rapport A+T/G+C et les caractères bactériologiques des Corynebacterium. Ann. Inst. Past. 104, 756.
12. BRENNAN, P.C., FRITZ, T.E. and FLYNN, R.J. (1969). Role of P. pneumotropica and M. pulmonis in Murine pneumonia. J. Bact. 97, 337.



13. BRENNER, D.J., MARTIN, M.A. and HOYER, E.H. (1967). Homology experiments among members of the Enterobacteriaceae. J. Bact. 94, 486.
14. BRENNER, D.J., FANNING, G.R., JOHNSON, K.E., CITARELLA, R.V. and FALKOW, S. (1969). Polynucleotide sequence relationships among members of the Enterobacteriaceae. J. Bact. 98, 637.
15. BURROWS, T.W. and GILLETT, W.A. (1966). The nutritional requirements of some Pasteurella species. J. gen. Microbiol. 45, 332.
16. CARMICHAEL, J.W., and SNEATH, P.H.A. (1969). Taxometric maps. Syst. Zool., 19, 402.
17. CATLIN, B.W. and CUNNINGHAM, L.S. (1958). Studies of extracellular and intracellular bacterial DNA. J. gen. Microbiol. 19, 522.
18. CATLIN, B.W. and CUNNINGHAM, L.S. (1961). Transforming activities and base contents of DNA preparations from various Neisseriae. J. gen. Microbiol. 26, 303.
19. CHARGAFF, E. (1950). Bacterial deoxypentoses of unusual composition. Am. chem. Soc. Jour. 72, 3825.
20. CHARGAFF, E. (1951). Structure, and function of nucleic acids as cell constituents. Fed. Proc. 10, 654.
21. CHARGAFF, E., LIPSHITZ, R., GREEN, C. and HODES, M.E. (1951). The composition of DNA from salmon sperm. J. biol. Chem. 192, 223.
22. CHARGAFF, E. and SAIDEL, H.F. (1949). On nucleoproteins of avian tubercle bacilli. J. biol. Chem. 177, 417.
23. CHARGAFF, E., VISCHER, E., DONIGER, R., GREEN, C. and MIRSANI, F. (1949). The compositions of the deoxypentose nucleic acids of calf thymus and spleen. J. biol. Chem. 177, 405.
24. CHARGAFF, E. and ZAMENOF, S. (1948). The isolation of highly polymerized deoxypentose nucleic acids from yeast cells. J. biol. Chem. 173, 327.
25. CLARKE, P.H. (1953). H<sub>2</sub>S production by bacteria. J. gen. Microbiol. 8, 397.
26. CLARKE, P.H. and COWAN, S.T. (1952). Biochemical methods for Bacteriology. J. gen. Microbiol. 6, 187.





27. COLWELL, R.R., CITARELLA, R.V. and RYMAN, I. (1965). Deoxyribonucleic acid base composition and Adansonian analysis of heterotrophic, aerobic Pseudomonads. *J. Bact.* 90, 1148.
28. COLWELL, R.R. and MANDEL, M. (1964). Adansonian analyses and DNA base composition of some Gram-negative bacteria. *J. Bact.* 87, 1412.
29. COLWELL, R.R. and MANDEL, M. (1965). Adansonian analysis and DNA base composition of *Serratia marcescens*. *J. Bact.* 89, 454.
30. COOK, G.T. (1950). A plate test for nitrate reduction. *J. clin. Path.* 3, 359.
31. COWAN, S.T. and STEELE, K.J. (1965). Manual for the identification of medical bacteria. Cambridge University Press.
32. CRAVERI, R., HILL, L.R., MANACHINI, P.L. and SILVESTRI, L.G. (1965). DNA base composition of thermophilic *Actinomycetes*. *J. gen. Microbiol.* 41, 335.
33. DAVIES, G.H.B. and PARK, R.W.A. (1962). Taxonomic study of certain bacteria currently classified as *Vibrio* species. *J. gen. Microbiol.* 27, 101.
34. DE LEY, J. (1964). Pseudomonads and related genera. *Ann. Rev. Microbiol.* 18, 17.
35. DE LEY, J. (1964). Effect of mutation on DNA composition of some bacteria. *Antonie van Leeuwenhoek*, 30, 281.
36. DE LEY, J., BERNAERTS, M., RASSEL, A. and GUILMOT, J. (1966). Approach to an improved taxonomy of the genus *Agrobacterium*. *J. gen. Microbiol.* 43, 7.
37. DE LEY, J. and RASSEL, A. (1965). DNA base composition, flagellation, and taxonomy of the genus *Rhizobium*. *J. gen. Microbiol.* 41, 85.
38. DE LEY, J. and SCHELL, J. (1963). DNA base composition of acetic acid bacteria. *J. gen. Microbiol.* 33, 243.
39. DE LEY, J. and VAN MUYLEM, J. (1963). Applications of DNA base ratios to bacterial taxonomy. *Antonie van Leeuwenhoek*, 29, 344.
40. DOTY, P., MCGILL, B.B. and RICE, S.A. (1959). Production of sonic fragments of DNA. *Proc. natn. Acad. Sci. U.S.A.* 44, 432.





41. DOWELL, V.R., LOPER, J. and HILL, E.O. (1964). Constancy of DNA base composition in the transition of Sphaerophorus necrophorus from bacilli to large bodies. J. Bact. 88, 1805.
42. EIKEN, M. (1958). Studies on an anaerobic, rod-shaped, Gram-negative microorganism Bacteroides corrodens N.sp. Acta path. microbiol. scand. 43, 404.
43. FEDOROVNA, L.S. (1964). Nucleic acid composition of DNA and RNA of the agent causing toxic bacteriosis of water melons. Mikrobiologiya, 33, 856.
44. FREDERICQ, E., OTH, A. and FONTAINE, F. (1961). The u.v. spectrum of DNAs and their constituents. J. molec. Biol. 3, 11.
45. FRONTALI, C., HILL, L.R. and SILVESTRI, L.G. (1965). DNA base composition of Streptomyces. J. gen. Microbiol. 38, 243.
46. GANDELMAN, B., ZAMENOF, S. and CHARGAFF, E. (1952). The deoxypentose nucleic acids of three strains of E. coli. Biochim. biophys. Acta 9, 399.
47. GARRITY, F.L., DETRICK, B. and KENNEDY, E.R. (1969). DNA base composition in the taxonomy of the genus Staphylococcus. J. Bact. 97, 557.
48. GASSER, F. and MANDEL, M. (1968). DNA base composition of the genus Lactobacillus. J. Bact. 96, 580.
49. GASSER, F. and SEBALD, M. (1966). Composition en bases nucleiques des Bacteries du genre Lactobacillus. Ann. Inst. Past. 110, 261.
50. GAUSE, G.C., LOSHKAREVA, N.P., ZBARSKY, I.B. and GAUSE, G.F. (1964). DNA base composition in certain bacteria and their mutants with impaired respiration. Nature, Lond. 203, 598.
51. HAAPALA, D.K., ROGUL, M., EVANS, L.B. and ALEXANDER, A.D. DNA base composition and homology studies of Leptospira. J. Bact. 98, 421.
52. HANSEN, A.J., WEEKS, O.B. and COLWELL, R.R. (1965). Taxonomy of Pseudomonas piscicida. J. Bact. 89, 1315.
53. HEBERLEIN, G.T., DE LEY, J. and TITJGAT, R. (1967). DNA homology and the taxonomy of the family Rhizobiaceae. J. Bact. 94, 116.
54. HENRIKSEN, S.D. (1969). Corroding bacteria from the respiratory tract. Acta path. microbiol. scand. 75, 91.



55. HENRIKSEN, S.D. and JYSSUM, K. (1960). A new variety of P. haemolytica from the human respiratory tract. Acta path. microbiol. scand. 50, 443.
56. HENRIKSEN, S.D. and JYSSUM, K. (1961). A study of some Pasteurella strains from the human respiratory tract. Acta path. microbiol. scand. 51, 354.
57. HILL, L.R. (1959). The Adansonian classification of the Staphylococci. J. gen. Microbiol. 20, 277.
58. HILL, L.R. (1965). An index to the DNA base composition of bacterial species. J. gen. Microbiol. 44, 419.
59. HOYER, B.H. and McCULLOUGH, N.B. (1968). Polynucleotide homologies of Brucella DNA. J. Bact. 95, 444.
60. HOYER, B.H. and McCULLOUGH, N.B. (1968). Homologies of DNA from Brucella ovis Canine Abortion Organisms, and other Brucella species. J. Bact. 96, 1783.
61. HUBALEK, Z. (1969). Numerical taxonomy of the genera Micrococcus (Cohn) and Sarcina(Goodsir). J. gen. Microbiol. 57, 349.
62. JOHNSON, J.L. and ORDAL, E.J. (1968). Parameters affecting DNA homology in bacterial taxonomy. J. Bact. 95, 893.
63. JONES, D.M. (1962). A Pasteurella-like organism from the respiratory tract. J. Path. Bacteriol. 83, 143.
64. JOSHI, J.G., GUILD, W.R. and HANDLER, P. (1963). The presence of two species of DNA in some species of Halobacter. J. molec. Biol. 6, 34.
65. KHAIRAT, O. (1967). Bacteroides corrodens isolated from Bacteremia. J. Path. Bact. 94, 29.
66. KINGSBURY, D.T. (1967). DNA homology among species of Neisseria. J. Bact. 94, 870.
67. KINGSBURY, D.T. and WEISS, E. (1968). Lack of DNA homology among species of Chlamydia. J. Bact. 96, 1421.
68. KNAPP, W. and THAL, E. (1963). Untersuchung über die kulturell-biochemischen, serologischen, tierexperimentellen und immunologischen Eigenschaften einer vorläufig Pasteurella X benannten Bakterienart. Zentbl. f. Bakt. I Abt. Orig. 190, 472.
69. KRIEG, R.E. and LOCKHART, W.R. (1966). Classification of the Enterobacteriaceae based on overall similarity. J. Bact. 92, 1275.





70. LEE, K.Y., WAHL, R. and BARBU, E. (1956). Contenu en bases puriques et pyrimidiques des acides desoxyribonucleiques des bacteries. Ann. Inst. Past. 91, 212.
71. LYNN, R.J. and SMITH, P.F. (1957). Nucleic acid content of PPLO from human sources. J. Bact. 74, 811.
72. MANDEL, M. (1965). DNA base composition in the genus Pseudomonas. J. gen. Microbiol. 43, 273.
73. MANDEL, M., BERGENDAHL, J.C. and PFENNIG, N. (1965). DNA base composition of isolates of the genus Chlorobium. J. Bact. 89, 917.
74. MANDEL, M. and LEADBETTER, E.R. (1965). DNA base composition of Myxobacteria. J. Bact. 90, 1795.
75. MARMUR, J. (1961). A procedure for the isolation of DNA from micro-organisms. J. molec. Biol. 3, 208.
76. MARMUR, J. and DOTY, P. (1959). Heterogeneity in DNA. I. Dependence on composition of the configurational stability of DNA. Nature, Lond. 183, 1427.
77. MARMUR, J. and DOTY, P. (1961). Thermal renaturation of DNA. J. molec. Biol. 3, 585.
78. MARMUR, J. and DOTY, P. (1962). Determination of the base composition of DNA from its thermal denaturation temperature. J. molec. Biol. 5, 109.
79. MARMUR, J. FALKOW, S. and MANDEL, M. (1963). New approaches to bacterial taxonomy. Ann. Rev. Microbiol. 17, 239.
80. MCCARTHY, B.J. and BOLTON, E.T. (1963). An approach to the measurement of genetic relatedness among organisms. Proc. natn. Acad. Sci. U.S.A. 50, 156.
81. McDONALD, W.C., FELKNER, I.C., TURETSKY, A. and MATNEY, T.S. (1963). Similarity in base composition of DNA from several strains of B. cereus and B. anthracis. J. Bact. 85, 1071.
82. MEYER, K.F. (1955). In Bacterial and mycotic infections of man. Ed. R.J. Dubos. 3rd. ed. Lippincott, Philadelphia.
83. MOLLARET, H.H. and LUCAS, A. (1965). Sur les particularités biochimiques, des souches de Yersinia enterocolitica isolées chez les lièvres. Ann. Inst. Past. 108, 127.
84. MRAZ, O. (1969). Vergleichende studie der Arten Actinobacillus ligniersi und P. haemolytica. I.A. ligniersi. Zentbl. f. Bakt. I Abt. Orig. 209, 212.





85. MRAZ, O. (1969). II. P. haemolytica. Zentbl. f. Bakt. I Abt. Orig. 209, 336.
86. MRAZ, O. (1969). III. Actinobacillus haemolyticus. Zentbl. f. Bakt. I Abt. Orig. 209, 349.
87. OWEN, R.J., HILL, L.R. & LAPAGE, S.P. (1969). Determination of DNA compositions from melting profiles in dilute buffers. Biopolymers, 7, 503.
88. PANOS, C. (1965). Cellular physiology during logarithmic growth of a Streptococcal L form. J. gen. Microbio. 39, 131.
89. PHILIPS, C.B. & OWEN, C.R. (1961). Comments on the nomenclature of the causative agent of Tularemia. Int. Bull. Bact. Nomen. Taxon. 11, 67.
90. POINDEXTER, J.S. (1964). Biological properties, and classification of Caulobacter group. Bact. Rev. 28, 231.
91. REICH, P.R., HYBNER, C.J., CHANOCK, R.M. & WIESSMAN, S.M. (1966). Relationships among Mycoplasma species of man. J. Bact. 92, 302.
92. RITTER, D.B. & GERLOFF, R.K. (1966). DNA hybridization among species of the genus Pasteurella. J. Bact. 92, 1838.
93. ROGUL, M., MCGEE, Z.A., WITTLER, R.G. & FALKOW, S. (1965). Nucleic acid homologies among selected bacteria, L-forms and Mycoplasma species. J. Bact. 9, 1200.
94. ROSS, P.D. & SCRUGGS, R.L. (1968). Viscosity study of DNA. Effect of simple salt concentration on viscosity of high molecular weight DNA. Biopolymers, 6, 1005.
95. ROSYPAL, S., ROSYPALOVÁ, A. & HOREJŠ, J. (1966). The classification of Micrococci and Staphylococci based on their DNA base composition and Adansonian analysis. J. gen. Microbiol. 44, 281.
96. SAUNDERS, G.F., CAMPBELL, L.L. & POSTGATE, J.R. (1964). Base composition of DNA of sulphate reducing bacteria, deduced from buoyant density measurement in CsCl. J. Bact. 87, 1073.
97. SCHILDKRAUT, C.L. & LIFSON, S. (1965). Dependence of melting temperature of DNA on salt concentration. Biopolymers, 3, 195.
98. SCHILDKRAUT, C.L., MARMUR, J. & DOTY, P. (1961). Formation of hybrid DNA molecules and their use in studies of DNA homologies. J. molec. Biol. 3, 595.
99. SCHILDKRAUT, C.L., MARMUR, J. & DOTY, P. (1962). Determination of the base composition of DNA from its buoyant density in caesium chloride. J. molec. Biol. 4, 430.



100. SEBALD, M., GASSER, F. & WERNER, H. (1965). Teneur GC% et classification. Application au groupe des Bifidobacteries et a quelques genres voisins. Ann. Inst. Past. 109, 251.
101. SEBALD, M. & VERON, M. (1963). Teneur en bases et classifications des Vibrions. Ann. Inst. Past. 105, 897.
102. SHAW, C. & CLARKE, P.H. (1955). Identification of Proteus and Providencia. J. gen. Microbiol. 13, 155.
103. SIGAL, N., SENEZ, J.C., LE GALL, J. & SEBALD, M. (1963). Base composition of DNA of sulphate-reducing bacteria. J. Bact. 85, 315.
104. SILVESTRI, L.G. & HILL, L.R. (1962). Agreement between DNA composition, and taxometric classification of Gram-positive cocci. J. Bact. 90, 136.
105. SMITH, J.D. & WYATT, G.R. (1951). Composition of some microbial deoxypentose nucleic acids. Bioc-em. J. 49, 144.
106. SOMERSON, N.L., REICH, P.R., WALLS, B.E., CHANOCK, R.M. & WEISSMAN, S.M. (1966). Genetic differentiation by nucleic acid homology. II Genotypic variations between two mycoplasma species. J. Bact. 92, 311.
107. SPIRIN, A.S., BELOZERSKY, A.N., SHUGYAEVA, W.V. & VANYUSHIN, B.F., (1957). A study of species specificity with respect to the nucleic acid of bacteria. Biokhimiya, 22, 744.
108. STANIER, R.Y., PALLERONI, N.J. & DOUDOROFF, M. (1966). The aerobic pseudomonads, a taxonomic study. J. gen. Microbiol. 43, 159.
109. SUEOKA, N. (1959). Analysis of DNA distribution in density gradient centrifugation. Proc. natn. Acad. Sci. U.S.A. 45, 1480.
110. SUEOKA, N. (1961). Variation and heterogeneity of base composition of DNA: a compilation of old and new data. J. molec. Biol. 3, 31.
111. SUEOKA, N., MARMUR, J. & DOTY, P. (1959). Heterogeneity in DNA. II. Dependence of the density of the DNA on the guanine+cytosine contents. Nature, Lond. 183, 1429.
112. TALBOT, J.M. & SNEATH, P.H.A. (1960). Taxonomic study of Pasteurella septica especially strains isolated from human sources. J. gen. Microbiol. 22, 303.
113. TONOMURA, B., MALKIN, R. & RABINOWITZ, J.C. (1965). DNA base composition of Clostridial species. J. Bact. 89, 1438.



114. VAN LOGHEM, J.J. (1946). La classification du Bacille pesteux. Ann. Inst. Past. 72, 975.
115. VERON, M. & SEBALD, M. (1964). Sur la teneur en bases de l'ADN et la position taxonomique de Vibrio ichthyodermis. Ann. Inst. 107, 442.
116. VISCHER, E. & CHARGAFF, E. (1948). Separation and quantitative estimation of purine and pyrimidine bases in minute amounts. J. biol. Chem. 176, 703.
117. VISCHER, E., ZAMENOF, S. & CHARGAFF, E. (1949). Deoxypentoses of avian tubercle bacilli, and yeast. J. biol. Chem. 177, 429.
118. WAYNE, L.G. & GROSS, W.M. (1968). Composition of DNA isolated from Mycobacteria. J. Bact. 92, 1372.
119. WEED, L.L. (1963). Effects of copper on B. subtilis. J. Bact. 85, 1003.
120. WEISSMAN, S.M., SOMERSON, N.L. & COLE, R.M. (1966). Genetic differentiation by nucleic acid homology. IV. Relationships among Lancefield groups and serotypes of Streptococci. J. Bact. 92, 1372.
121. YANOFSKY, C. (1967). Ann. Rev. Genetics, 1, 117.
122. ZAMENOF, S., BRAWERMAN, G. & CHARGAFF, E. (1952). On the deoxy-pentose nucleic acid from several microorganisms. Biochem. biophys. Acta 9, 402.























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